Mechanisms of Biotransformation of Methoxylated and Hydroxylated Polybrominated Diphenyl Ethers

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PREFACE

Chapter 2 of this thesis has been published in journal of Environmental Science and Technology*. Chapter 3 of this thesis has been organized as a manuscript that has been submitted for publication to Aquatic Toxicology. For this reason, some repetition of introductory and methodological material is unavoidsdable.

***** Wan, Y., Liu, F., Wiseman, S., Zhang, X., Chang, H., Hecker, M., Jones, P. D., Lam, M. H. W., Giesy, J. P. 2010c. Interconversion of hydroxylated and methoxylated polybrominated diphenyl ethers in Japanese medaka. *Environ. Sci. Technol.* 44, 8729-873

ABSTRACT

Polybrominated diphenyl ethers (PBDEs) were the most commonly used non-chemically bound brominated flame retardants (BFRs). The PBDEs as well as their methoxylated- (MeO-) and hydroxylated- (OH-) analogs are distributed in environmental matrices worldwide and have been detected in several organisms. There is concern over the occurrence of the OH-BDEs because they have greater toxicities relative to the PBDEs, including neurotoxicity, immunotoxicity, disruption of energy metabolism, and reproductive and endocrine disruption. Relationships among PBDEs, OH-BDEs and MeO-BDEs are unclear. While MeO-BDEs and OH-BDEs are naturally occurring in the marine environment, it has been reported that OH-BDEs are formed by biotransformation of PBDEs. However, in these studies, the OH-BDEs occurred at trace levels, from <0.01-1% of exposed PBDEs levels. A recent study by Wan et al. (2009) suggested that instead of PBDEs, *in vitro,* naturally occurring MeO-BDEs are precursors of OH-BDEs. However, there is no evidence from *in vivo* studies that the MeO-BDEs are transformed to OH-BDEs. Further, the enzyme(s) that catalyze the transformation of MeO-BDEs to OH-BDEs were unknown. To further demonstrate *in vivo,* biotransformation relationships among these structurally related compounds dietary accumulation, maternal transfer, and tissue distribution of PBDEs, MeO-BDEs and OH-BDEs and their transformation products were investigated in sexually mature Japanese medaka (*Oryzias latipes*). Medaka were exposed to BDE-47, 6-OH-BDE-47, and 6-MeO-BDE-47 through their diet for 14 days. Concentrations of all compounds were quantified in livers and whole carcass (minus livers) of female medaka as well as in eggs. Significant concentrations of 6-OH-BDE-47 were quantified in medaka exposed to 6-MeO-

BDE-47. Significant concentrations of 6-MeO-BDE-47 were also detected in fish exposed to 6-OH-BDE-47. However, 6-MeO-BDE-47 was not observed when microsomes from livers of medaka were exposed to 6-OH-BDE-47. OH-PBDEs and MeO-PBDEs were not detected in medaka exposed to BDE-47. Similar results were demonstrated in eggs from female medaka. Furthermore, as hypothesized, concentrations of BDE-47, 6-MeO-BDE-47 and 6-OH-BDE-47 in medaka eggs increased during exposure. Therefore, this study demonstrated *in vivo* biotransformation of 6-MeO-BDE-47 to 6-OH-BDE-47 as a primary pathway, while conversion from BDE-47 to 6-OH-BDE-47 did not occur.

The enzyme(s) that catalyze transformation of MeO-BDEs to OH-BDEs was characterized in liver of rainbow trout (*Oncorhynchus mykiss*). Significantly greater concentrations of 6-OH-BDE-47 were detected in microsomes than were observed in S9 fractions exposed to 6-MeO-BDE-47, which suggests that biotransformation of 6-MeO-BDE-47 to 6-OH-BDE-47 is localized in microsomes. The requirement for the co-factor NADPH further confirmed the catalysis by phase I enzymes in this biotransformation reaction. Non-significant transformation of 6-MeO-BDE-47 to 6-OH-BDE-47 in microsomes isolated from livers of rainbow trout exposed to the aryl-hydrocarbon receptor (AhR) agonist β-napthoflavone (βNF) compared to unexposed rainbow trout indicated that members of the CYP 1 family enzymes were not involved in this transformation. Inhibitors of CYP enzymes, clotrimazole (CL), 1-benzylimidazole (BI) and gestodene (GE) and an anti-CYP 3A antibody were used to further investigate the enzymes involved. Transformation of 6-MeO-BDE-47 to 6-OH-BDE-47 was significantly inhibited by the broad spectrum inhibitors of CYP enzymes, CL and BI. However, neither the CYP 3A inhibitor GE nor the anti-CYP 3A antibody

significantly altered the rate of transformation in microsomes exposed to 6-MeO-BDE-47. Therefore, transformation of 6-MeO-BDE-47 to 6-OH-BDE-47 is more likely to be catalyzed by enzymes of the CYP 2 family.

Because activities of CYP enzymes can be different among fishes, it was hypothesized that transformation of 6-MeO-BDE-47 to 6-OH-BDE-47 would also differ among fishes. Differences of transformation from 6-MeO-BDE-47 to 6-OH-BDE-47 among rainbow trout, white sturgeon (*Acipenser transmontanus*) and goldfish (*Carassius auratus*) were investigated. Microsomes isolated from these three species were incubated with 6-MeO-BDE-47 for 0.5, 1, 2, 6 and 24 h. The greatest concentrations of 6-OH-BDE-47 were detected in microsomes isolated from livers of rainbow trout, followed by goldfish and white sturgeon. The initial rate of transformation for rainbow trout was significantly greater than that of goldfish and white sturgeon, while goldfish also had a significantly greater initial rate than white sturgeon. The final concentration of transformation product after 24 h exposure for rainbow trout was greater than that of goldfish and white sturgeon. Similarly, the final concentration of OH-BDE-47 in goldfish was significantly greater than white sturgeon. In addition, differences in the concentrations of OH-BDEs determined in different species of fish could be due to differences in CYP-catalyzed transformation of MeO-BDEs to OH-BDEs.

Taken together, the results are consistent with the conclusion that naturally occurring MeO-BDEs, and not the synthetic PBDEs, are the primary source of the biologically potent OH-BDEs. An enzyme of the CYP 2 family was suggested to transform 6-MeO-BDE-47 to 6-OH-BDE-47. It was demonstrated that the degree of biotransformation of MeO-BDEs to

OH-BDEs is dependent on species of fish. Therefore, since the OH-BDEs have greater toxicity than PBDEs, different species exposed to the same concentration of MeO-BDEs may be differentially impacted by this exposure due to differences in the capacity to convert MeO-BDEs to OH-BDEs.

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LIST OF ABBREVIATIONS

ACN	Acetonitrile
AhR	aryl hydrocarbon receptor
ANOVA	analysis of variance
AR	androgen receptor
ATRF	Aquatic Toxicology Research Facility
BDE-17	2,2',4-Tribromodiphenyl ether
BDE-28	2,4,4'-Tribromodiphenyl ether
BDE-47	2,2',4,4'-Tetrabromodiphenyl ether
BDE-49	2,2',4,5'-Tetrabromodiphenyl ether
BDE-68	2,3',4,5'-Tetrabromodiphenyl ether
BDE-99	2,2',4,4',5-Pentabromodiphenyl ether
BDE-100	2,2',4,4',6-Pentabromodiphenyl ether
BDE-119	2,3',4,4',6-Pentabromodiphenyl ether
BDE-153	2,2',4,4',5,5'-Hexabromodiphenyl ether
BDE-154	2,2',4,4',5,6'-Hexabromodiphenyl ether
BFRs	brominated flame retardants
BI	1-benzylimidazole
CL	clotrimazole
CYP	cytochrome P450
d	day (s)
DCM	dichloromethane
Deca-BDEs	decabromodiphenyl ethers

DI	deiodinase
DTT	dithiothreitol
E/F	ratios between egg and whole fish
E/L	ratios between egg and liver
E2	17β-estrogen
EDTA	ethylenediaminetetraacetic acid
ER	17β-estrogen receptor
EROD	Ethoxyresorufin *O*-deethylase
EU	European Union
GE	Gestodene
h	hour (s)
HCl	hydrochloric acid
HRGC	high-resolution gas chromatography
HRMS	high-resolution mass spectrometry
HSD	Honestly Significant Differences
L/F	ratios between liver and whole fish
LC	liquid chromatography
M/P	ratios between transformation products and their parent compounds
MDL	method detection limits
MeO-	methoxylayted
min	minute (s)
MRM	multi-reaction monitoring
mRNA	messenger RNA
MS/MS	tandem mass spectrometry

MTBE	methyl *tert*-butyl ether
NADPH	nicotinamide adenine dinucleotide phosphate
Octa-BDEs	octabromodiphenyl ethers
OH-	hydroxylated
PBDEs	polybrominated diphenyl ethers
PCBs	polychlorinated biphenyls
Penta-BDEs	pentabromodiphenyl ethers
QA/QC	Quality Assurance and Quality Control
rT3	reverse thyronine
SD	standard deviation
SIM	Selective Ion-Monitoring
T	Testosterone
T3	3, 3', 5-triiodo-thyronine
T4	thyroxine
TBG	thyroxine binding globulin
TCDD	2, 3, 7, 8-tetrachloro-dibenzo-p-dioxin
TR	thyroid hormone receptors
TTR	transthyretin
U of S-AREB	University of Saskatchewan Animal Research Ethics Board
βNF	β-napthoflavone

CHAPTER 1

GENERAL INTRODUCTION

1.1 Introduction to Polybrominated Diphenyl Ethers

1.1.1 Sources and Concentrations in the Environment

Brominated flame retardants (BFRs) have been used in electronic equipment, textiles and plastics for fire resistance because they are the most cost-effective flame retardants. Some brominated flame retardants are chemically bound to plastics or textiles, while others are non-chemically bound additives mixed into polymers and therefore can leach from their product into the environment (de Wit, 2002). Total global production of all BFRs in 1992 was estimated to be approximately 150,000 tons with approximately 40% used in North America, 30% in the Far East and 25% in Europe (Arias, 1992; KEMI, 1994). The most commonly used group of non-chemically bound BFRs are the polybrominated diphenyl ethers (PBDEs). Three commercial formulations, pentabromodiphenyl ethers (Penta-BDEs), octabromodiphenyl ethers (Octa-BDEs) and decabromodiphenyl ethers (Deca-BDEs), were produced for use in commercial products (Costa et al., 2008). The general structure of a PBDE consists of two brominated aromatic rings connected by an ether bond (Figure 1.1). There are 209 possible PBDE congeners that differ by the number and positions of the bromine on the two aromatic rings. PBDEs are structurally similar to Polychlorinated

biphenyls (PCBs) and dioxins, including 2, 3, 7, 8-tetrachloro-dibenzo-p-dioxin (TCDD), and therefore they have similar properties (WHO, 1994). PBDEs are lipophilic and resistant to degradation. The $\log_{10}$ of the octanol-water partition coefficient ($\log K_{ow}$) ranges from 5 - 8.9 depending on the degree of bromination (Watanabe and Tatsukawa, 1990).

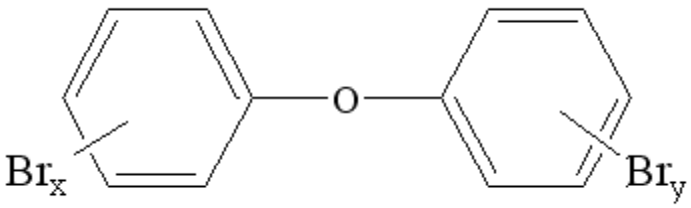

Figure 1.1 Structure of polybrominated diphenyl ethers where x + y = 1 to 10.

PBDEs undergo long-range atmospheric transport, which results in world-wide distribution of these compounds in various environmental matrices, including air, water, soils and sediments. Similar to PCBs, the lesser halogenated congeners of PBDEs are transported by a “grasshopper” effect such that they tend to condence out in the Arctic (Gouin et al., 2004; Moon et al., 2007; Nöel et al., 2009; Wang et al., 2005), while PBDEs with more bromines, due to their lesser volatility, undergo more local-scale transport (Wania and Dugani, 2003). Because the C-Br bond is less strong than is the C-Cl bond, once in the environment PBDEs, are more easily degraded than are polychlorinated biphenyls (PCBs) (Hooper and McDonald, 2000). However, due to relatively continuous releases, PBDEs are still ubiquitous in the

global environment. In Asia, PBDEs have been detected in air, aquatic sediments and human milk (Watanabe et al., 1995; Ohta et al., 2000). In Europe, PBDEs have also been found in sediments, fish and air (Allchin et al., 1999; de Wit, 2002). Similar to Asia and Europe, measureable amounts of PBDEs exist in urban, rural and remote regions of North American, including the Great Lakes region (Dodder et al., 2000). Concentrations of PBDEs in tissues of humans are greater in North America than other areas (Patterson et al., 2000; Ryan and Patry, 2000). Based on rates of bioaccumulation of PBDEs in the Canadian Arctic between 1981 and 2000, by 2050 PBDEs will replace PCBs as the most prevalent organohalogen compound in Canadian Arctic ringed seals (Ikonomou et al., 2002).

Concentrations of PBDEs in sediments, fishs or marine mammals began increasing measurably at the end of the 1980s (Noren and Meironyte, 1998, 2000). In the early 1990s, concern over increasing concentrations of PBDEs in wildlife and humans, and the potential for PBDEs to significantly affect organism health led some European countries to place a "voluntary" ban on the use of the Penta-BDE formulation (Schecter et al., 2003). Due to the results of risk assessments, in 2004 the European Union (EU) officially banned production, use or import of penta- and octa-brominated diphenylethers (Penta-BDE and Octa-BDE). This action resulted in a "voluntary" ban on production of PBDEs in some states in America (Betts, 2008). In 2003, the Governor of California signed a bill to phase out the use of these two formulations, effective January 1, 2008 (Anonymous, 2003). However, a recent review indicated that concentrations of PBDEs, including those used in the Penta-BDE and Octa-BDE formulations are still increasing in the environment (Eriksson et al., 2001). PBDEs are

still being emitted from primary production facilities, manufacturing plants that incorporate PBDEs in their products as well as from landfills where PBDEs leach out from the polymer of discarded consumer products.

1.1.2 Biotransformation of PBDEs

Reduction (reductive debromination) and oxidation (hydroxylation) are two of the primary pathways for biotransformation of PBDEs (Hakk et al., 2002, 2006, 2009; Orn and Klasson-Wehler, 1998; Qiu et al., 2007; Staskal et al., 2006). The presence of NADPH in assays using microsomes isolated form livers of common carp (*Cyprinus carpio*), did not significantly increase debromination of 2,2',4,4',5-pentabromodiphenyl ether (BDE-99) (Benedict et al., 2007). Non-NADPH dependent debromination of BDE-99 to 2,2',4,4'-tetrabromodiphenyl ether (BDE-47) was also demonstrated in microsomes isolated from livers of Chinook salmon (*Onchorhynchus tshawytscha*) (Browne et al., 2009). However, significantly less debromination of BDE-99 was observed in microsomes spiked with reverse thyronine (rT3). This result is consistent with the hypothesis that the primary pathway for debromination of PBDEs is catalyzed by thyroid hormone deiodinases (DI), and not cytochrome P450 (P450) enzymes (Benedict et al., 2007). Furthermore, omission of dithiothreitol (DTT) from the incubation mixture resulted in significantly less debromination of BDE-99, a result which is consistent with microsomal reductase(s) or deiodinase enzymes catalysing debromination of PBDEs (Browne et al., 2009). As a consequence of

debromination, BDE-47 is the most abundant PBDE congener in the environment (La Guardia et al., 2007).

It has been suggested that PBDEs can be biotransformed into hydroxylated (OH-) BDEs. In contrast to debromination, P450-mediated formation of epoxide intermediates has been suggested to be the first step for biotransformation of PBDEs to hydroxylated forms of brominated diphenylethers (OH-BDE) (Hakk et al., 2009). Biotransformation of PBDEs to OH-BDEs was reported to be NADPH-dependant, which indicated that the process is catalyzed by a P450 enzyme (McKinney et al. 2006a). Selected PBDE congeners were individually incubated with hepatic microsomes from rates induced with either phenobarbital (CYP2B inducer), β-napthaflavone (CYP1A inducer), or clofibrate (CYP4A3 inducer) (Meerts et al., 2000). The incubation mixtures were tested by a competitive thyroxine-transthyretin (T4-TTR) binding assay. The results suggested that biotransformation products generated via CYP2B enzymes of lesser brominated PBDEs were able to compete with T4 for binding to TTR. Because OH-BDEs bind with greater avidity to TTR than PBDEs that are not hydroxylated, the results indicated that CYP2B mediates hydroxylation of PBDEs. Most recently, involvement of different P450 enzymes in the oxidative metabolism of BDE-47 and BDE-99 by microsomes isolated from livers of rats was investigated (Erratico et al. 2011). When BDE-47 was incubated with microsomes isolated from livers of rats that had been exposed to the P450 2B inducer, phenobarbitol, five OH-BDE biotransformation products were detected with the most prevalent being 4'-hydroxy-2,2',4,5'-tetrabromodiphenyl ether (4’-OH-BDE-49) and 3-hydroxy-2,2',4,4'-tetrabromodiphenyl ether (3-OH-BDE-47). When

BDE-99 was incubated with microsomes isolated from livers of rats exposed to the P450 3A inducer dexamethasone, seven OH-BDEs were detected with 4-hydroxy-2,2',3,4',5-pentabromodiphenyl ether (4-OH-BDE-90) being the most prevalent. Furthermore, results of studies that used recombinant CYP enzymes of rats, suggested that P450 2A2 and P450 3A1 were the major enzymes responsible for hydroxylation of BDE-47 and BDE-99, respectively. However, formation of 4'-OH-BDE-49 and 6'-hydroxy-2,2',4,4',5-pentabromodiphenyl ether (6'-OH-BDE-99) were due to catalysis by P450 1A1 while generation of 3-OH-BDE-47 and 4-OH-BDE-90 were primarily generated due to activity of CYP 3A1.

Biotransformation of PBDEs was compared among several teleost fishes (Johnson and Olson, 2001). Rainbow trout, mountain whitefish (*Prosopium cylindraceum*), white sucker (*Catosstomus commersoni*) and common carp (*Cyprinus carpio*) were captured in the same freshwater area in Washington, US. Rainbow trout and mountain whitefish were always found to have greater concentrations of PBDEs than did white sucker or common carp. It was concluded that the former two species have greater metabolic activities than the later two. In another study different concentrations of 6 PBDEs and 10 OH-BDEs were identified in 13 species of fish collected from the Detroit River (Valters et al., 2005). Significant differences of the total concentrations of OH-BDEs and of 6-hydroxy-2,2',4,4'-tetrabromodiphenyl ether (6-OH-BDE-47) were observed in blood plasma from different fishes (Valters et al., 2005). Specifically, concentrations of both PBDEs and OH-BDEs in plasma of common carp were greater than that of lake sturgeon (*Acipenser fulvescens*) (Valters et al., 2005).

1.2 Structural Analogs of PBDEs - Hydroxylated and Methoxylated BDEs

Hydroxylated (OH-) (Figure 1.2a) and methoxylayted (MeO-) BDEs (Figure 1.2b) are structurally similar to PBDEs. Unlike PBDEs, neither OH-BDEs nor MeO-BDEs were intentionally produced by industry (Haglund et al., 1997; Malmvärn et al., 2005; Valters et al., 2005). Both OH-BDEs and MeO-BDEs were originally detected in marine sponges (Anjaneyulu et al., 1996; Handayani et al., 1997). The OH-BDEs and MeO-BDEs have also been detected in marine organisms such as algae, fishes and mussels (Marsh et al., 2004; Malmvärn et al., 2005, 2008). Greater concentrations of OH-BDEs and MeO-BDEs have also been detected in higher organisms including in the plasma of fishes from Lake Ontario and the Baltic Sea (de la Torre et al., 2009; Asplund et al., 1999; Marsh et al., 2004), and whales from the Atlantic Ocean (Van Bavel et al., 2001). MeO-BDEs were present in freshwater fishes (Kierkegaard et al., 1999), marine fishes (Haglund et al., 1997; Asplund et al., 1999; Marsh et al., 2004; Sinkkonen et al., 2004), seals (Haglund et al., 1997) and birds from the Baltic Sea (Olsson et al., 2000; Sinkkonen et al., 2004).

In addition to their presence in wildlife, OH-BDEs and MeO-BDEs have been identified in health products, including commercial fish oil (Haglund et al., 1997; Asplund et al., 1999). Most recently, Covaci et al. (2007) found concentrations of MeO-BDEs in fish oil dietary supplements that were 3 times greater than concentrations of PBDEs. Due to the dietary intake of fish products, it is not surprising that both OH-BDEs and MeO-BDEs were found in humans. OH-BDEs have been observed in humans from all over the world, including Sweden, Japan, Nicaragua, The Netherlands, United States, Spain, China and South Korea (Hovander

et al., 2002; Kawashiro et al., 2008; Athanasiadou et al., 2008; Meijer et al., 2008; Qiu et al., 2009; Lacorte and Ikonomou, 2009; Yu et al., 2010; Wan et al., 2010a). OH-BDEs have been detected in human milk (Lacorte and Ikonomou, 2009) and blood plasma from placentas (Kawashiro et al., 2008; Qiu et al., 2009; Wan et al., 2010a) which could mean that infants and developing fetuses are exposed to these chemicals.

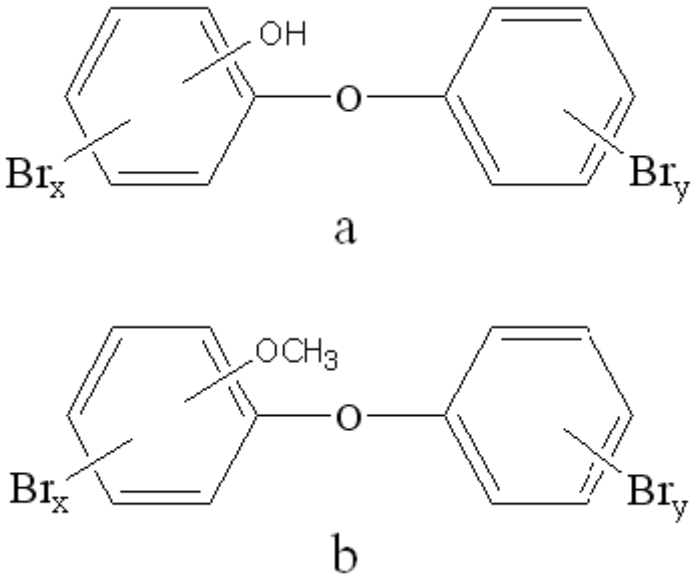

Figure 1.2 Structure of hydroxylated polybrominated diphenyl ethers (a) and methoxylated polybrominated diphenyl ethers (b).

1.3 Sources of OH-BDEs and MeO-BDEs

1.3.1 Natural Occurrence of OH-BDEs and MeO-BDEs

Organohalogen compounds are ubiquitous in the environment. Many organohalogen compounds are anthropogenic, in particular the fluorinated and chlorinated compounds, but most brominated compounds are from natural sources (Gribble, 2003). For example, polybrominated diphenyl methanes and brominated phenols are naturally produced by several species of red macroalga in waters along the Swedish West coast (Pedersen et al., 1974, 1978). Of the greater than 4000 natural organohalogen compdounds, approximately 1950 are organobromines, almost all of which are produced by marine organisms including bacteria, plants or animals (Gribble, 2003). Although chlorine is more abundant in the marine environment than is bromine, due to the lesser energy of C-Br bond in comparison with C-Cl bond and the greater concentration of bromoperoxidase enzymes compared to chloroperoxidase enzymes in marine organisms (Gribble, 1998, 2000), more organobromine compounds are produced by marine organisms (Wan et al., 2010b).

To date, several OH-BDEs (2′-OH-BDE-28, 2′-OH-BDE-68, 2-OH-BDE-123, 6′-OH-BDE-17, 6-OH-BDE-47, 6′-OH-BDE-49, 6-OH-BDE-85, 6-OH-BDE-90, 6-OH-BDE-99, 6-OH-BDE-137) and MeO-BDEs (6-MeO-BDE-47, 2′-MeO-BDE-68, 2′-MeO-BDE-28, 6-MeO-BDE-85, and 6-MeO-BDE-137) are known to be natural compounds produced by marine organisms such as the marine sponge *Dysidea herbacea* or its associated filamentous cyanobacterium *Oscillatoria spongeliae*, red alga (*Ceramium tenuicorne*), or green

alga (*Cladophora fascicularis*) (Bowden et al., 2000; Fu et al., 1995; Handayani et al., 1997; Malmvärn et al., 2005, 2008). The most abundant natural products are 6-OH-BDE-47 and 6-methoxy-2,2',4,4'-tetrabromodiphenyl ether (6-MeO-BDE-47). In each of these natural occurring compounds the hydroxyl or methoxyl group is located in *ortho* position relative to the diphenyl ether bond (reviewed in Wiseman et al., 2011). C-14 stable isotope analysis of MeO-BDE-47 and MeO-BDE-68 isolated from North Atlantic True's beaked whales (*Mesoplodon mirus*) proved that these are natural products (Teuten et al., 2005). Detection of *ortho*-substituted MeO-BDEs and OH-BDEs in whale oil collected in 1921, which predates manufacture of PBDEs, further demonstrated that these compounds are naturally occurring (Teuten and Reddy, 2007).

1.3.2 OH-BDEs as Products of Biotransformation

Because MeO-BDEs and OH-BDEs occur in marine algae and sponges, their biomagnification through the aquatic food chain is generally accepted as one source of these compounds in organisms. However, the occurrence of OH-BDEs and MeO-BDEs in organisms inhabiting freshwater and terrestrial systems far from marine environments suggested that other sources of these compounds might exist. The results of several studies have suggested that PBDEs are precursors of OH-BDEs. When microsomes isolated form beluga whale (*Delphinapterus leucas*) were exposed to 4,4'-dibromodiphenyl ether (BDE-15), a single OH-BDE congener was detected (McKinney et al., 2006a). OH-BDEs have also been

detected in human hepatocytes (Stapleton et al., 2009) and in rat microsomes (Erratico et al., 2010) incubated with BDE-99. When Kierkegaard et al. (2001) exposed northern pike (*Esox lucius*) to BDE-47 via the diet six different OH-BDEs were detected. OH-BDEs also have been detected in tissues of rats and mice exposed to various PBDEs (Hakk et al., 2002, 2006, 2009; Chen et al., 2006; Staskal et al., 2006; Marsh et al., 2006; Qiu et al., 2007; Malmberg et al., 2005).

OH-BDEs were not detected in all studies of PBDEs. OH-BDE transformation products were not detected in common carp (Stapleton et al., 2006) or Chinook salmon (Browne et al., 2009) exposed *in vivo* to BDE-99. Similarly, OH-BDEs were not detected in microsomes isolated from beluga whale exposed to either 2,4,4'-tribromodiphenyl ether (BDE-28) or BDE-47 (McKinney et al., 2006a). A recent study failed to detect OH-BDEs in microsomes of White-leghorn Chicken (*Gallus gallus*) or rainbow trout (*Oncorhynchus mykiss*) with S9 fractions from Sprague Dawley rats exposed to either BDE-99 or a PBDE mixture (Wan et al., 2009). Concentrations of OH-BDEs were less than the limit of quantification in Chinese sturgeon (*Acipenser sinensis*) microsomes that were incubated with BDE-47, -99, -154 or -183 (Zhang et al., 2010).

Because OH-BDEs are not always produced following exposure to PBDEs there is doubt that PBDEs can be biotransformed to OH-BDEs. In fact, in all studies where OH-BDEs were detected following either *in vivo* or *in vitro* exposures to PBDE the reported concentrations of the OH-BDEs always ranged from less than 0.1% to 1% of the concentrations of the PBDEs at the beginning of the exposure. A recent study by Wan et al. (2009) suggested that instead

of PBDEs, the naturally occurring MeO-BDEs are the precursor of OH-BDEs. When microsomes isolated from livers of rainbow trout, chicken and rat were incubated with either BDE-99, 6-MeO-BDE-47 or 6-OH-BDE-47 the concentrations of OH-BDEs were less than the detection limit in all BDE-99 exposed samples, and MeO-BDEs or PBDEs were not detected in microsomes exposed to 6-OH-BDE-47. However, 6-OH-BDE-47 was detected in microsomes incubated with 6-MeO-BDE-47, and the concentrations were approximately 10% of concentrations of the 6-MeO-BDE-47. Similar results were obtained when microsomes were incubated with mixtures of PBDEs, OH-BDEs, or MeO-BDEs (Wan et al., 2009). Concentrations of PBDEs, OH-BDEs and MeO-BDEs in wildlife also suggest that MeO-BDEs, not the PBDEs are the source of OH-BDEs found in organisms worldwide (Wan et al., 2009; Zhang et al., 2010). A strong, positive correlation between concentrations of OH-BDEs and MeO-BDEs in tuna (*Katsuwonus pelamis*), five albatrosses (*Thalassarche chlororhynchos*, *Phoebetria palpebrata, T. chrysostoma, T. cauta, and T. melanophrys*), and polar bear (*Ursus maritimus*) was determined. However, no correlation between PBDEs and OH-BDEs was demonstrated (Wan et al., 2009). Similarly, Zhang et al. (2010) elucidated a strong correlation between 6-OH-BDE-47 and 6-MeO-BDE-47, but not BDE-47 and 6-OH-BDE-47 or BDE-47 and 6-MeO-BDE-47 in Chinese sturgeon in the Yangtze River.

1.4 Toxicity of PBDEs, OH-BDEs and MeO-BDEs

Toxicities of PBDEs have been extensively studied. PBDEs exhibit a range of toxicities including neurotoxicity, immunotoxicity, disruption of energy metabolism, cancer, and reproductive and endocrine disruption (Vonderheide et al., 2008). Studies have also focused on the toxicity of the OH-BDEs and MeO-BDEs. What has become clear from these studies is that in almost all systems the OH-BDEs have greater toxicities than either the MeO-BDEs or PBDEs. The greater toxicities of OH-BDEs relative to the MeO-BDEs or PBDEs are reviewed here by comparing their endocrine disrupting effects.

1.4.1 Thyroid Hormone Disruption

The thyroid hormone thyroxine (T4) is produced by the thyroid gland and secreted into the blood where it binds to the transport protein transthyretin (TTR) and is carried to the target tissues. In these tissues T4 is deiodinated to the biologically active form 3, 3', 5-triiodo-thyronine (T3). The T3 then binds to either the alpha (TR-α) or beta (TR-β) form of the thyroid hormone receptor (TR) and the T3-TR complex binds to response elements located in the promoter regions of target genes to regulate relevant biological effects (Forrest and Vennstrom, 2000). This system can be affected by xenobiotics, including PBDEs and OH-BDEs (Brouwer et al., 1998; Brucker-Davis, 1998; Meerts et al., 2000, 2001; Qiu et al., 2007).

In vitro studies have demonstrated that PBDEs and OH-BDEs can affect the thyroid hormone system by acting as agonists of TTR (Meerts et al., 2000, 2001; Qiu et al., 2007). This effect is attributed to their structural similarity to T4 and T3. To compare the binding affinity of PBDEs and OH-BDEs to TTR, PBDEs were incubated with microsomes and comparisons of the ability of biotransformed and non-biotransformed PBDEs to disrupt binding of either T3 or T4 to TTR was determined. The results demonstrated that "metabolic activation" of PBDEs, which the authors suggested was the generation of OH-BDEs, resulted in generation of compounds with greater binding affinities for TTR than either T3 or T4 (Meerts et al., 2000, 2001; Qiu et al., 2007).

In addition to competing with the binding of T3 and T4 to TTR, inhibition of binding of T3 to TR has been reported for OH-BDEs (Marsh et al., 1998). Synthetic T4 and T3 analogues, as well as two other OH-BDEs, 4-hydroxydiphenyl ether and 4'-OH-BDE-30 were tested for the binding affinity to TR. The T3 analogue had the strongest binding affinity to the TR, while the binding affinity of the T4 analogue was approximately 30% of the T3 analogue. The two OH-BDEs bound to the TR, but with lesser affinity than both of T3 and T4 analogues (Marsh et al., 1998). The affinity of PBDEs or OH-BDEs for TR was also examined by Kitamura et al. (2008). 4-OH-BDE-90 and 3-OH-BDE-47 inhibited the binding of T3 to TR in the concentration range of 1×10^{-6} M to1×10^{-4} M. However, none of PBDEs used in that study had an affinity for TR. Great affinity to TR was demonstrated for 3- and 4-OH-BDEs with bromines in both positions flanking the hydroxyl group (Kitamura et al., 2008). These results demonstrated that OH-BDEs have a greater ability than PBDEs to influence the

expression of T3-responsive genes. OH-BDE-TR complex binds to the response element gene to influence regulation of thyroid hormone-dependent genes is suggested by these results.

As reviewed in section 1.2, OH-BDEs have been detected in human breast milk, maternal plasma and even fetal serum. In some cases the concentrations of OH-BDEs in the offspring were greater than concentrations measured in the mother (Qiu et al., 2009; Wan et al., 2010a). This might have significant detrimental effects with respect to development of the fetus. It is known that T4 cannot be produced by the fetus during the first trimester of development (Calvo et al., 2002). Therefore, all T4 is of maternal origin and is able to cross the placental membrane via TTR (Landers et al., 2009). As mentioned previously, OH-BDEs have high affinity of binding to TTR, which may cause the accumulation of OH-BDEs in fetus and the disruption of T4 transportation from the mother to the developing fetuses.

1.4.2 Estrogenicity and Androgenicity

PBDEs, MeO-BDEs and OH-BDEs have the ability to disrupt synthesis of 17β-estrogen (E2) and testosterone (T), and to interfere with E2 and T signaling pathways. The PBDE congeners BDE-47 and BDE-99, which are found extensively in environmental samples, can act as estrogen receptor agonists *in vitro*. However, they have lesser potency compared to E2, with the maximum response less than 10% of that for E2 (Harrahy et al., 2004). Eleven lesser brominated PBDEs activated E2 receptor (ER) signaling in the T47D-Kbluc reporter gene

assay (Meerts et al., 2001). However, the estrogenic potency of these PBDE congeners was less than that of E2. In contrast to the lesser brominated PBDEs, three PBDEs containing more Br atoms, displayed anti-E2 activity. Similar results were demonstrated by Hamers et al. (2006), who investigated the estrogen potency of selective brominated PBDEs.

The number and positions of bromine atoms determines the estrogenicity/anti-estrogenicity of PBDEs. Only those PBDEs with two *ortho* bromine atoms on one phenyl ring and no bromine atoms on *ortho-meta* or *meta* positions on the other ring, or at least one *para* bromine atom have estrogenic potency (Meerts et al., 2001). However, the presence of the OH- group(s) on the OH-BDEs imparts greater estrogenicity on these compounds than the un-hydroxylated PBDEs (Meerts et al., 2001). In fact, all OH-BDEs have greater estrogenic potency than the corresponding non-hydroxylated PBDEs (Meerts et al., 2001). The naturally occurring 6-OH-BDE-47 has greater anti-estrogenic potency than all the more brominated PBDEs (Hamers et al., 2006).

Androgenic hormones such as T can bind to the androgen receptor (AR) in the cytoplasm and then be translocated into the nucleus to regulate gene expression. However, AR activities can be disrupted by xenobiotics such as PBDEs (Kojima et al., 2009). PBDEs, OH-BDEs and MeO-BDEs were also shown to have effects on synthesis of E2 and T. At 3-weeks post-birth a dose-dependent decrease in concentrations of E2 was determined in male rat pups exposed to lesser (1 mg/g bw/day) and greater (10 mg/g bw/day) doses of BDE-99 during gestational days 10 to 18 (Lilienthal et al., 2006). Concentrations of T in the greater dose group remained significantly less than that of control group even after 160 days of exposure. MeO-BDEs

affect T and E2 synthesis in the H295R cell line, but the effects were dependent on the structure of the MeO-BDE (He et al., 2008). Greater synthesis of E2 was observed in cells exposed to 6-methoxy-2,2',3,4,4'-pentabromodiphenyl ether (6-MeO-BDE-85) but only slightly greater synthesis of T was identified. In contrast, there was lesser production of E2 and no effects on production of T in cells exposed to 2′-MeO-BDE-23. Synthesis of T was greater in cells exposed to 6-MeO-BDE-137. Overall, greater effects on mRNA abundance of steroidogenic enzymes were demonstrated in H295R cells exposed to MeO-BDEs compared to OH-BDEs. By using a reporter gene assay, Liu et al. (2011) recently demonstrated anti-estrogenic and anti-androgenic potency of several OH-BDEs and BDE-47. Specifically, 4'-OH-BDE-17 exhibited the greatest anti-estrogenic potency, followed by 6-OH-BDE-47, 2'-OH-BDE-28 and BDE-47. The greatest anti-androgenic potency was exhibited by 6-OH-BDE-47, followed by 4'OH-BDE-17, and 2'OH-BDE-28.

1.5 Summary of Research Objectives

1.5.1 Determine the Biotransformation of MeO-BDEs to OH-BDEs *in vivo*

Previous *in vitro* studies have demonstrated that MeO-BDEs, and not PBDEs, are the major precursors of the more biological potent OH-BDEs (Wan et al., 2009). However, biotransformation relationships among PBDEs, OH-BDEs and MeO-BDEs have not been investigated *in vivo*. To investigate biotransformation of PBDEs, OH-BDEs and MeO-BDEs *in vivo*, Japanese medaka will be exposed to BDE-47, 6-OH-BDE-47 ad 6-MeO-BDE-47 via

the diet and concentrations of products of biotransformation will be determined in livers and whole carcasses from each group. In addition, concentrations of metabolites will be determined in eggs collected from female fish throughout the exposure period to investigate the maternal transfer of parent compounds and their metabolites.

Testable Null Hypothesis 1:

Neither BDE-47 nor 6-MeO-BDE-47 will be biotransformed to OH-BDEs in Japanese medaka exposed to these chemicals via their diet.

Testable Null Hypothesis 2:

Concentrations of exposed chemicals BDE-47, 6-MeO-BDE-47 or 6-OH-BDE-47 in Japanese medaka eggs will not increase over the duration of the exposure.

1.5.2 Identify the Specific Enzyme(s) which Biotransform MeO-BDEs to OH-BDEs

MeO-BDEs can be biotransformed to OH-BDEs *in vitro* (Wan et al., 2009), but the specific enzyme(s) involved in this biotransformation are unknown. Therefore, a study to investigate the mechanisms of biotransformation of MeO-BDEs to OH-BDEs will be conducted. Using microsomes isolated from livers of rainbow trout as a model system, the subcellular fraction where the biotransformation reactions occurred will be identified, and the specific enzymes involved in the reaction will be investigated using inhibitors of different families of cytochrome P450 enzymes. Involvement of the aryl hydrocarbon receptor (AhR)

will be investigated by incubating 6-MeO-BDE-47 with microsomes isolated form livers of rainbow trout exposed to the AhR agonist β-napthoflavone (βNF).

Testable Null Hypothesis 3:

Biotransformation of 6-MeO-BDE-47 to 6-OH-BDE-47 will not be different in different subcellular fractions isolated from livers form rainbow trout.

Testable Null Hypothesis 4:

Biotransformation of 6-MeO-BDE-47 to 6-OH-BDE-47 will not be different among microsomes isolated from control rainbow trout and rainbow trout exposed to βNF.

Testable Null Hypothesis 5:

Biotransformation of 6-MeO-BDE-47 to 6-OH-BDE-47 will not be different among microsomes isolated from livers of rainbow trout that are incubated with inhibitors of different P450 enzymes.

1.5.3 Species comparison of transformation from MeO-BDEs to OH-BDEs

In addition, differences in transformation of MeO-BDEs to OH-BDEs in different fishes were investigated to determine whether differences in concentrations of OH-BDEs in different species could be due to differences in the transformation of MeO-BDEs to OH-BDEs.

Testable Null Hypothesis 6:

Concentrations of OH-BDEs will not increase over the course of the incubation. Furthermore, the rates for biotransformation of 6-MeO-BDE-47 to 6-OH-BDE-47 will not be different among different species of fish.

CHAPTER 2

INTERCONVERSION OF HYDROXYLATED AND METHOXYLATED POLYBROMINATED DIPHENYL ETHERS IN JAPANESE MEDAKA

Abstract

Polybrominated diphenyl ethers (PBDEs), hydroxylated (OH-) and methoxylated (MeO-) PBDEs have been widely detected in aquatic environment. However, relationships among these structurally related compounds in exposed organisms have been unclear. To elucidate biotransformation relationships among BDE-47, 6-OH-BDE-47, and 6-MeO-BDE-47, dietary accumulation, maternal transfer, and tissue distribution of these compounds and their transformation products were investigated in sexually mature Japanese medaka (*Oryzias latipes*). In addition, transformation of each compound was determined *in vitro* using liver microsomes of medaka. OH-PBDEs and MeO-PBDEs were not detected in fish exposed to the parent BDE-47. However, significant concentrations of 6-OH-BDE-47 were detected in medaka or microsomes exposed to 6-MeO-BDE-47. Significant concentrations of 6-MeO-BDE-47 were also measured in fish exposed to 6-OH-BDE-47, but 6-MeO-BDE-47 was not detected in microsomes exposed to 6-OH-BDE-47. Similar patterns of transformation products were observed in medaka eggs during exposure. This study presents direct, *in vivo* evidence of biotransformation of 6-MeO-BDE-47 to 6-OH-BDE-47. In addition, this is the first study to demonstrate biotransformation of 6-OH-BDE-47 to 6-MeO-BDE-47. Demethylation of 6-MeO-BDE-47 was the primary transformation pathway leading to

formation of 6-OH-BDE-47 in medaka, while the previously hypothesized formation of OH-PBDEs from synthetic BDE-47 did not occur. Biotransformation products formed in adult female medaka were transferred to eggs.

Keywords: Metabolism, Maternal transfer, Eggs, Embryo toxicity, Fish, Tissue distribution

2.1 Introduction

Over the course of the past decade brominated flame retardants (BFRs) have emerged as persistent organic pollutants of concern (Hites, 2004). Among the different classes of BFRs, polybrominated diphenyl ethers (PBDEs) have received the greatest attention, mostly due to their widespread use, ubiquitous environmental distribution, and bioaccumulation potential (Hites, 2004). Recently focus has shifted to structural analogues of PBDEs, such as hydroxylated (OH-) and methoxylated (MeO-) PBDEs. From evidence in marine sponges, algae and mussels in some marine systems, it is strongly suggested that these compounds are widely detected in the marine environment and their concentrations in some instances exceed those of PBDEs (Unson et al., 1994; Teuten et al., 2005; Malmvärn et al., 2005). The occurrence of OH-PBDEs is of particular interest since they exhibit greater toxicity than PBDEs or MeO-PBDEs (Boxtel et al., 2008; Marchesini et al., 2008). Effects of OH-PBDEs on organisms include disruption of thyroid hormone homeostasis, disruption of oxidative phosphorylation, altered estradiol synthesis, and neurotoxicity (Meerts et al., 2001; Canton et al., 2005; Dingemans et al., 2008; Boxtel et al., 2008; Morgado et al., 2007). Relatively great ratios of transfer of OH-PBDEs from parent to offspring have been reported for pregnant women (Wan et al., 2010a) and wild fish (Chinese sturgeon) (Zhang et al., 2010).

Currently there is debate over the origin of OH-PBDEs and MeO-PBDEs (Wan et al., 2009; Fernie and Letcher, 2010). It has been suggested that ortho- substituted OH-PBDEs and MeO-PBDEs are formed from naturally occurring compounds in marine ecosystems (Unson et al., 1994; Teuten et al., 2005). Two abundant congeners of MeO-PBDEs (6-MeO-BDE-47 and 2'-MeO-BDE-68) have been reported to be natural products of marine organisms (Teuten et al., 2005). Similarly, ortho- substituted OH-PBDEs are produced naturally by marine algae or associated microorganisms (Unson et al., 1994; Malmvärn et al.,

2005). MeO-PBDEs have been suggested to be formed via methylation of OH-PBDEs. This pathway was hypothesized based on knowledge of bacterial methylation of phenols in the environment (Teuten et al., 2005; Allard et al., 1987; Haglund et al., 1997). Alternatively, the similarity in structure between these compounds and synthetic PBDEs has led to suggestions that meta-/para- substituted OH-PBDEs and MeO-PBDEs could originate from biotransformation of synthetic PBDEs (Malmberg et al., 2005; Hamers et al., 2008; Stapleton et al., 2009). Several studies have demonstrated that OH-PBDEs can be biotransformation products of PBDEs in fish, rat, and human cell cultures (Malmberg et al., 2005; Hamers et al., 2008; Stapleton et al., 2009). However, concentrations of OH-PBDEs detected in laboratory studies were extremely low compared to the ratios of concentrations of OH-PBDEs and MeO-PBDEs to PBDEs in marine organisms (Zhang et al., 2010; Wan et al., 2009; Malmberg et al., 2005; Hamers et al., 2008; Stapleton et al., 2009). Based on these results the occurrence of OH- and MeO-PBDEs in wild organisms cannot be explained by formation from synthetic PBDE alone. This information is consistent with the existence of sources of both OH-PBDEs and MeO-PBDEs other than synthetic PBDEs. Based on *in vitro* exposures using rainbow trout, chicken, and rat microsomes it was reported that demethylation of naturally occurring MeO-PBDEs is an important contributor for the occurrence of OH-PBDEs found in wildlife from remote areas (Wan et al., 2009).

While the results of *in vitro* studies provided insight into biotransformation relationship(s) between PBDEs, MeO-PBDEs and OH-PBDEs, these were still to be confirmed *in vivo*. In this study, relationships among BDE-47, 6-MeO-BDE-47 and 6-OH-BDE-47 were explored in sexually mature Japanese medaka (*Oryzias latipes*). Medaka were exposed to each chemical through their diet. Relationships among the target chemicals were assessed in liver, residual carcass (body without liver) and eggs. Furthermore, accumulation into embryos and maternal transfer of the three target compounds and their transformation products were

investigated by measuring their concentrations in eggs of exposed adult medaka. Finally, based on the data generated during this study and a thorough review of the available literature, origin and plausible transformation pathways of these brominated compounds are proposed and discussed. Sources of the various PBDEs as well as OH- and MeO-PBDEs have important implications for risk assessment and how exposures can be controlled.

2.2 Materials and methods

2.2.1 Chemicals and Standards

MeO-PBDEs (6-MeO-BDE-17, 4-MeO-BDE-17, 2'-MeO-BDE-68, 6-MeO-BDE-47, 5-MeO-BDE-47 and 4'-MeO-BDE-49) and OH-PBDEs (3-OH-BDE-47, 5-OH-BDE-47, 6-OH-BDE-47, 4'-OH-BDE-49 and 6'-OH-BDE-17) were synthesized in the Department of Biology and Chemistry, City University of Hong Kong, Hong Kong, China. PBDEs (BDE-28, BDE-47, BDE-99, BDE-100, BDE-119, BDE-154 and BDE-153) and ^{13}C-PBDEs were obtained from AccuStandard (New Haven, Connecticut, USA). All compounds were determined to be >98% pure by high-resolution gas chromatograph interfaced to a high-resolution mass spectrometer (HRGC-HRMS). Dichloromethane (DCM), n-hexane, nonane, methyl *tert*-butyl ether (MTBE), acetone, acetonitrile and methanol were pesticide residue grade and were obtained from OmniSolv (EM Science, Lawrence, KS, USA). Silica gel (60-100 mesh size), formic acid, hydrochloric acid (37%, A.C.S. reagent), 2-propanol and dansyl chloride were obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.2.2 Animals

Male and female wild-type *O. latipes* were obtained from a stock maintained at the aquatic culture unit at the US Environmental Protection Agency Mid-Continent Ecology Division (Duluth, MN, USA). Individuals were maintained at the Aquatic Toxicology Research Facility (ATRF) in the Toxicology Centre, University of Saskatchewan (Saskatoon, SK, Canada), by use of previously described methods. Medaka were cultured in flow-through tanks under conditions that facilitated breeding (23-24 °C; 16:8 light/dark cycle) and were fed once daily to satiety. All protocols were approved by the University of Saskatchewan Animal Research Ethics Board (U of S-AREB).

2.2.3 Preparation of Diet

Commercial fish food (Nutrafin Basix Staple Food, Rolf C. Hagen Inc. Canada) was ground with a mortar and pestle, weighed into a glass flask, and spiked with known amounts of each target compound (BDE-47, 6-MeO-BDE-47 or 6-OH-BDE-47) dissolved in 150 mL of acetone. The flask was shaken for 30 min to ensure thorough mixing of the food and chemicals. Following mixing, the contents of the flasks were concentrated to dryness in a rotary-evaporator. The resulting spiked food was air-dried for approximately 3 h in a fume hood. An identical protocol was used to prepare the acetone-spiked "control" food. Concentrations of BDE-47, 6-MeO-BDE-47 and 6-OH-BDE-47 in the stock standards and food samples were analyzed (Table 2.1) using methods described below.

Table 2.1 Concentrations of 6-OH-BDE-47, 6-MeO-BDE-47, and BDE-47 in spiked food (ng/g dry weight) and stock standard solutions (ng/mL).

	6-OH-BDE-47	6-MeO-BDE-47	BDE-47
Control food	<0.02	0.1	<1.6
6-OH-BDE-47 spiked food	900	0.2	15
6-MeO-BDE-47 spiked food	<0.02	8,000	28.3
BDE-47 spiked food	<0.02	0.2	21,000
6-OH-BDE-47 stock solutions	1500,000	4,300	1,900
6-MeO-BDE-47 stock solutions	<0.8	1300,000	4,800
BDE-47 stock solutions	<0.8	<2.0	50,000

Underlined numbers are concentrations of parent compounds. Numbers that are not underlined are the concentrations of impurities.

2.2.4 Feeding Study

Prior to initiation of exposure to PBDE or PBDE transformation products, 5-month-old medaka (mean weight: 0.60±0.08 g, 8 females and 4 males per tank) were randomly assigned to 10-L tanks containing 6 L of dechlorinated tap water. Animals were allowed to acclimate to the experimental tanks for 3 d prior to the initiation of exposure. Each day approximately 50% of the water volume in the tanks was replaced; two replicate tanks were used for each treatment. Water temperatures were maintained at 23-24 ^{o}C during the course of the experiment.

Medaka were fed diets of food spiked with BDE-47, 6-MeO-BDE-47, or 6-OH-BDE-47 or the carrier solvent acetone alone (vehicle control). This method provided an effective means of administering the chemicals without stressing the fish. Dietary exposure was deemed a more realistic route of exposure than intraperitoneal injections (Gamperl et al., 1994). Fish were fed approximately 2% of their average body weight per day, half the daily

food was provided in the morning and the other half in the afternoon. All fish displayed vigorous eating behavior and the food was completely consumed in each tank.

Medaka were exposed to each target chemical for 14 d, and eggs were collected each morning (day 0 to 14) during the exposure period. After collection eggs from each individual tank were rinsed with Nanopure® (Barnsted) water and gently dried on Kimwipes®. The mass of each egg was determined, and then eggs from each exposure were composited and stored separately at -20 °C until analysis. On day 14, following egg collection, six female fish were sampled from each treatment tank and sacrificed for determination of target chemicals. The liver was dissected from each fish and both liver and the liver-free residual carcass were weighed and stored at -20 °C until analysis.

To confirm the results of the *in vivo* feeding study, transformation of BDE-47, 6-MeO-BDE47, and 6-OH-BDE-47 was investigated using microsomes isolated from liver of female medaka obtained from the same breeding colony as those animals used in the feeding study, according to the method of Kennedy and Jones (1994). The details of the methods used for microsomal incubations are described by Wan et al. (2009).

2.2.5 In vitro Biotransformation

To confirm the results of the *in vivo* feeding study, transformation of BDE-47, 6-MeO-BDE47, and 6-OH-BDE-47 was investigated using microsomes isolated from female medaka liver according to the method of Kennedy and Jones (1994). The details of the methods used for microsomal incubations are described elsewhere (Wan et al., 2009). Briefly, reactions were performed in 0.1 M NaH_2PO_4 buffer (pH 7.4) containing 1 mM ethylenediaminetetraacetic acid (EDTA), 10 mM dithiothreitol (DTT), and 100 µM NADPH.

The final reaction volume was 250 µL and contained 125 µL of microsomes and 10 µL of exposure chemicals. The concentration of the chemical in the final reaction mixture was 2 µg/mL. Incubations without chemicals and without microsomes were used as negative controls to assess the presence of background contaminants and the possibility of non-enzyme mediated changes in chemical structure. After incubation, the samples were extracted immediately for quantification of residues and their transformation products.

2.2.6 Sample Extraction and Cleanup

The methods used to quantify 6-OH-BDE-47 have been published previously (Chang et al., 2010). For this study quantifications of BDE-47 and 6-MeO-BDE-47 were incorporated in the same method. Liver (~ 0.02 g), egg (~ 0.2 g), fish food (~ 0.2 g), and microsomal incubation mixture (250 µL) were homogenized and transferred into amber tubes. After spiking with surrogate recovery standards, 2 mL of Nanopure® water (18 MΩ), 50 µL hydrochloric acid (HCl, 37%), and 3 mL of 3-propanol were added to the samples. Samples were extracted three times with 3 mL hexane/ methyl *tert*-butyl ether (MTBE) (1:1; v/v). To remove residual acid, extracts were washed four times with 4 mL aliquots of pure water. Samples were concentrated and dried under nitrogen. For whole fish (about 0.6 g), the extraction process was the same as described above, except that the amount of each solvent used was 10-fold greater to accommodate the greater mass.

Dried residues were dissolved in 200 µL of aqueous sodium bicarbonate (100 mmol/L, pH adjusted to 10.5 with sodium hydroxide) and 200 µL of dansyl chloride (1 mg/mL in acetone) was added. After vortex mixing for 1 min, the samples were incubated at 60 °C for 5 min. Then, 1 mL pure water and 3 × 3 mL of hexane were added, and the organic solvent

layer was removed and transferred onto a silica gel column (60-100 mesh size) for fractionation. The silica gel column was wet packed with 4 g silica gel and 4 g sodium sulfate. After application of the extract, the column was eluted with 15 mL hexane/DCM (1:1, v/v), and then 20 mL DCM. The first fraction was evaporated to dryness and reconstituted with 50 µL nonane for high-resolution gas chromatography combined with high-resolution mass spectrometry (HRGC-HRMS) analysis of PBDEs (BDE-28, BDE-47, BDE-99, BDE-100, BDE-119, BDE-154 and BDE-153) and MeO-PBDEs (6-MeO-BDE-17, 4-MeO-BDE-17, 2' -MeO-BDE-68, 6-MeO-BDE-47, 5-MeO-BDE-47 and 4' -MeO-BDE-49). The second fraction was evaporated to dryness and reconstituted with 50 µL of acetonitrile:water (60:40) for liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis of OH-PBDEs (3-OH-BDE-47, 5-OH-BDE-47, 6-OH-BDE-47, 4'-OH-BDE-49 and OH-triBDEs).

2.2.7 Instrumental Analysis

Identification and quantification of BDE-47 and 6-MeO-BDE-47 were performed using a Hewlett-Packard 5890 series II high-resolution gas chromatography (HRGC) interfaced to a Micromass® Autospec® high-resolution mass spectrometry (HRMS) (Micromass®, Beverly, MD). Chromatographic separation was achieved by use of a DB-5MS fused silica capillary column for all target compounds (30 m length, 0.25 mm ID, 0.1 µm film, Agilent, Carlsbad, CA) and helium was used as the carrier gas. The mass spectrometer was operated in a Selective Ion-Monitoring (SIM) mode. Resolution of all reference peaks in all time windows was greater than 7,000. The injector temperature was held at 285 °C and the ion source was kept at 285 °C. The electron ionization energy was 37 eV and the ion current was 750 µA. The temperature program was from 110 °C (10 min) to 250 °C at a rate of 25 °C/min, then

increased to 260 °C at a rate of 1.5 °C/min, and then to 323 °C (15 min) at a rate of 25 °C/min.

Quantification of 6-OH-BDE-47 was conducted using an Agilent 1200 series high performance liquid chromatography system (Santa Clara, CA, USA) connected to an API 3000 triple-quadrupole tandem mass spectrometry (MS/MS) system (PE Sciex, Concord, ON, Canada). An XBridge C18 column (100 × 2.1 mm, 3.5 µm particle size) from Waters (Milford, MA, USA) was used for chromatographic separation at room temperature. Injection volume was 20 µL. The mobile phase, consisting of acetonitrile (Solvent A) and 0.1% formic acid in water (Solvent B), was used with a gradient elution of A:B = 60:40 (0-1 min) to 95:5 (1-15 min) and 95:5 (15-22 min) at a flow rate of 0.25 mL/min. Analytes were detected using a mass spectrometer equipped with a turbo ion spray source operated in the positive multi-reaction monitoring (MRM) mode. All the source and instrument parameters were optimized by infusing the purified dansyl derivatives of analytes into the mass spectrometer. Optimal MS conditions were: ion spray voltage 3750 V, curtain gas (N_2) 8, nebulizer gas (N_2) 12, collision gas (N_2) 10, turbo ion spray probe temperature 475 °C.

2.2.8 Quality Assurance and Quality Control (QA/QC).

To avoid cross contamination among treatments, equipment was changed after processing each treatment. To confirm the purities of the chemicals used in this study, concentrations of target compounds were analyzed in spiked food and stock standard solutions (Table 2.1). To avoid sample contamination during chemical analysis, all equipment was rinsed repeatedly with acetone and hexane. A laboratory blank was incorporated in the analytical procedures for every batch of 10 samples. Concentrations of all

congeners were quantified by the internal standard isotope-dilution method using mean relative response factors determined from standard calibration runs. BDE-47 and 6-MeO-BDE-47 were quantified in sample extracts relative to ^{13}C-BDE-47, and 6-OH-BDE-47 was quantified relative to 6'-OH-BDE-17. Recoveries of ^{13}C-BDE-47 and 6'-OH-BDE-17 were 82±26% and 72±28%, respectively, in all samples, and the concentrations of the analytes were recovery-corrected. The method detection limits (MDL) were defined as three times of the standard deviation in the blank samples, in which BDE-47 was detected. The MDLs for the other compounds, which were not detected in blank samples, were set to the instrumental minimum detectable amounts. Detection limits were 0.02 ng/g ww, 0.05 ng/g ww, and 1.6 ng/g ww for 6-OH-BDE-47, 6-MeO-BDE-47 and BDE-47, respectively, in analyzed samples. For those results less than the MDL, half of the MDL was assigned to avoid missing values in statistical analyses.

2.2.9 Data Analysis

All statistical analyses were performed using SPSS 11.0 (SPSS Inc., Chicago, IL, USA). Results are reported as mean ± standard deviation (SD). Differences between two groups were analyzed by the non-parametric Wilcoxon signed rank sum test. Differences in concentrations of target compounds among treatments were compared using one-way analysis of variance (ANOVA). Levene's test was used to check the equality of variances (the value of significance is less than 0.05). Where variances were equal, data were analyzed by the F test. Where the equality of variances could not be assumed, Welch's and Brown-Forsythe's robust tests were used to perform one-way ANOVA analysis. Multiple paired comparisons were used to determine which means differed from one another. Tukey's Honestly Significant Differences (HSD) was used where variances were presumed to be

equal, and the Games-Howell test was used where equality of variances could not be assumed. Results were considered significant when *p* values were less than 0.05.

2.3 Results

2.3.1 Purity of Stock Solutions and Food Preparations

6-OH-BDE-47 was not present as impurity in stock standard solutions of BDE-47 or 6-MeO-BDE-47. Negligible concentration of 6-MeO-BDE-47 (4.3 ng/g) was detected as impurity (0.002%) in the 6-OH-BDE-47 stock standard solution (Table 2.1). BDE-47 was detected as impurity in both stock solutions of 6-OH-BDE-47 (0.12%) and 6-MeO-BDE-47 (0.36%). The concentrations of the parent compounds in prepared food were 900, 8000 and 2100 ng/g dry weight for 6-OH-BDE-47, 6-MeO-BDE-47 and BDE-47, respectively. 6-OH-BDE-47 was not present as an impurity in either the BDE-47 or the 6-MeO-BDE-47 spiked food. Concentrations of BDE-47 in the 6-MeO-BDE-47 and 6-OH-BDE-47 food preparations were 28.3 ng/g and 15 ng/g, respectively. Trace amounts of 6-MeO-BDE-47 (0.1-0.2 ng/g) were detected in the control (acetone spiked), 6-OH-BDE-47, and BDE-47 food preparations. The presence of 6-MeO-BDE-47 and BDE-47 as impurities in stock solutions and prepared food did not affect conclusions drawn from the studies.

2.3.2 Concentrations and Distributions of Target Compounds in Medaka

Of all the screened compounds, only BDE-47, 6-MeO-BDE-47, and 6-OH-BDE-47 were detected in tissues fom medaka exposed separately to individual chemicals for two weeks (Figure 2.1, Figure 2.2). Concentrations of parent compounds were 5500±2200 ng/g ww,

3200±2300 ng/g ww and 190±330 ng/g ww for BDE-47, 6-MeO-BDE-47 and 6-OH-BDE-47, respectively, in livers of exposed medaka. Concentrations of parent compounds were 5200±1400 ng/g ww, 2600±970 ng/g ww and 14±5.9 ng/g ww for BDE-47, 6-MeO-BDE-47, and 6-OH-BDE-47, respectively, in liver-free residual carcass of medaka. 6-OH-BDE-47 was only detected as a transformation product in medaka (liver: 63±71 ng/g ww; liver-free residual carcass: 9.3±4.2 ng/g ww) exposed to 6-MeO-BDE-47 (Figure 2.1a). Concentrations of 6-MeO-BDE-47 (liver: 14±7.6 ng/g ww, liver-free residual carcass: 12±3.6 ng/g ww) detected in female medaka exposed to 6-OH-BDE-47 were significantly greater than concentration in medaka that were unexposed or exposed to BDE-47 (p<0.01, Figure 2.1b). Comparable concentrations of BDE-47 were observed in female medaka exposed to 6-MeO-BDE-47 (liver: 25±19 ng/g ww, liver-free residual carcass: 14±5.6 ng/g ww) and 6-OH-BDE-47 (liver: 19±20 ng/g ww, liver-free residual carcass: 9.4±10 ng/g ww) (Figure 2.1 c).

Distributions of each of the three chemicals either as parent compound or transformation product were different among liver, egg, and whole fish (Table 2.2). Concentration ratios of 6-OH-BDE-47 between liver and whole fish (L/F) were 6.1-6.9, which was greater than those of 6-MeO-BDE-47 (1.2) and BDE-47 (1.1). Concentration ratios between egg and liver (E/L) were 0.74-0.76 and 0.93 for 6-MeO-BDE-47 and BDE-47, respectively, which were comparable to those between egg and whole fish (E/F) of 6-MeO-BDE-47 (0.62 and 0.72) and BDE-47 (0.92). But greater E/F ratios (0.34 and 1.8) compared to E/L ratios (0.23 and 0.59) were observed for 6-OH-BDE-47. Significantly greater maternal transfer ratios were observed for 6-OH-BDE-47 (0.59-1.8) as parent compound than as a transformation product (0.23-0.34).

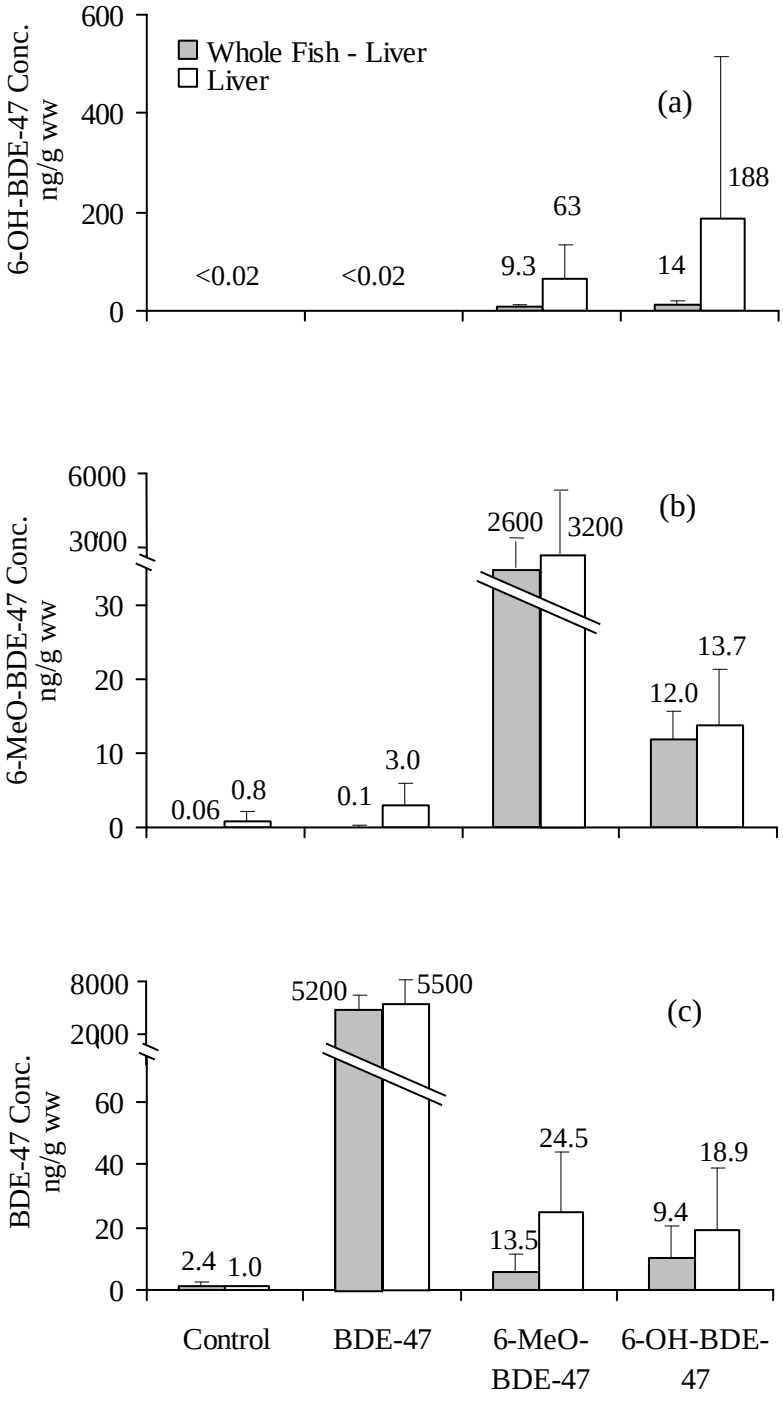

Figure 2.1 Concentrations of BDE-47, 6-MeO-BDE-47, and 6-OH-BDE-47 in liver (Liver) and liver free residual carcass (Whole Fish-Liver) tissue from female Japanese medaka after 14 d of dietary exposure to feed spiked with either solvent (control), BDE-47, 6-MeO-BDE-47, or 6-OH-BDE-47. The numbers above the error bars are the average concentrations of target compounds (ng/g ww).

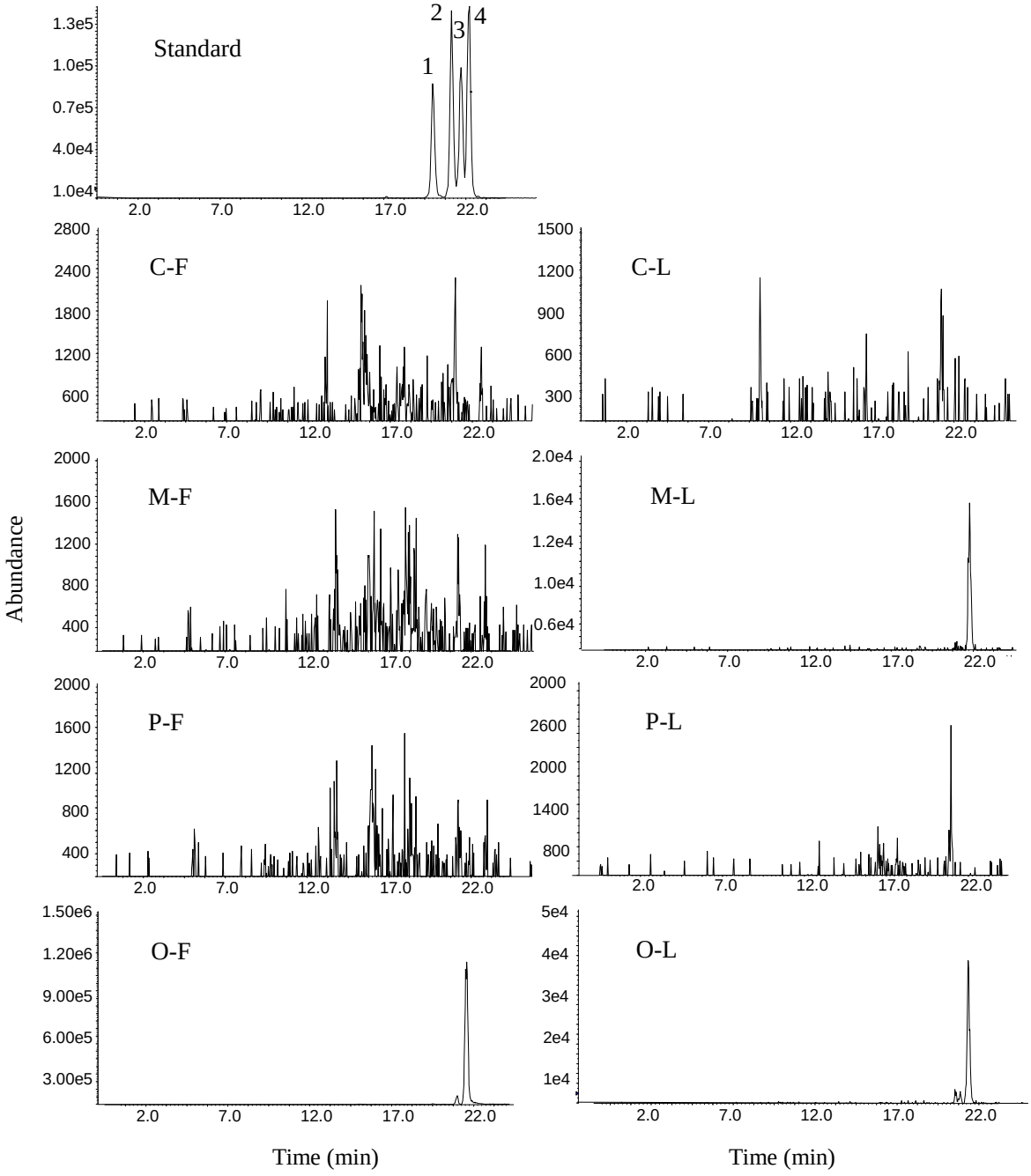

Figure 2.2 LC-MS/MS MRM chromatographic profiles of OH-Tetra-BDEs detected in prepared food and medaka livers after 14-day exposure. Standard: OH-Tetra-BDEs standard solution (5 ng/ml), 3-OH-BDE-47 (1), 5-OH-BDE-47 (2), 6-OH-BDE-47 (3), 4'-OH-BDE-49 (4); C-F: control food; M-F: 6-MeO-BDE-47 spiked food; P-F: BDE-47 spiked food; O-F: 6-OH-BDE-47 spiked food; C-L: liver of medaka in control group; M-L: liver of medaka exposed to 6-MeO-BDE-47; P-L: liver of medaka exposed to BDE-47; O-L: liver of medaka exposed to 6-OH-BDE-47.

Table 2.2 Concentration ratios (liver/whole fish, egg/whole fish and egg/liver) of exposed chemicals and metabolites in female Japanese medaka after a 14 d dietary exposure to feed spiked with either BDE-47, 6-MeO-BDE-47 or 6-OH-BDE-47.

Exposure groups	6-OH-BDE-47		6-MeO-BDE-47		BDE-47	
	Exposed chemical 6-OH-BDE-47	Metabolite 6-MeO-BDE-47	Exposed chemical 6-MeO-BDE-47	Metabolite 6-OH-BDE-47	Exposed chemical BDE-47	Metabolite -
Liver/Whole fish (L/F)	6.9±7.6	1.2±0.5	1.2±0.7	6.1±5.9	1.1±0.5	-
Egg/ Whole fish (E/F)	1.8±0.93	0.72±0.22	0.62±0.41	0.34±0.12	0.92±0.23	-
Egg/ Liver (E/L)	0.59±0.44	0.76±0.40	0.74±0.71	0.23±0.35	0.93±0.31	-

2.3.3 Accumulation in Eggs

Each of the parent compounds and their biotranformation products were detected in eggs collected during the exposure period. Daily egg mass production rates were 0.17 ± 0.05, 0.14±0.04, 0.19±0.03 and 0.18±0.06 g ww for control, BDE-47-exposed, 6-MeO-BDE-47-exposed and 6-OH-BDE-47-exposed during the exposure period, respectively. The slopes of the accumulation trend lines, as a function of exposure time during the initial six days of the exposure, were 1.94, 1.44, and 0.73 for 6-MeO-BDE-47, BDE-47 and 6-OH-BDE-47, respectively (Figure 2.3). Accumulation of 6-OH-BDE-47 and 6-MeO-BDE-47 in eggs reached steady-state on days six and twelve, respectively, but steady-state was not observed for BDE-47 (Figure 2.3). 6-OH-BDE-47, as a transformation product, was only detected in eggs of medaka exposed to 6-MeO-BDE-47 with concentrations ranging from 0.5 to 4.1 ng/g ww during the exposure period (Figure 2.4 (a)). Concentrations of 6-MeO-BDE-47, as a transformation product of 6-OH-BDE-47, increased in a time-dependent manner over the entire course of the study (Figure 2.4 (b)). Concentration ratios between transformation products and their parent compounds (M/P) in eggs of medaka exposed to 6-MeO-BDE-47 (6-OH-BDE47/6-MeO-BDE47: 0.003±0.002) were less than those of fish exposed to 6-OH-BDE-47 (6-MeO-BDE47/6-OH-BDE47: 0.057±0.030). The M/P ratios in medaka eggs were significantly less than those in liver and liver-free residual carcass in each treatment (6-OH-BDE47/6-MeO-BDE47: liver = 0.021±0.022, liver-free residual carcass = 0.005±0.006; 6-MeO-BDE47/6-OH-BDE47: liver = 0.273±0.249, liver-free residual carcass = 0.998±0.466).

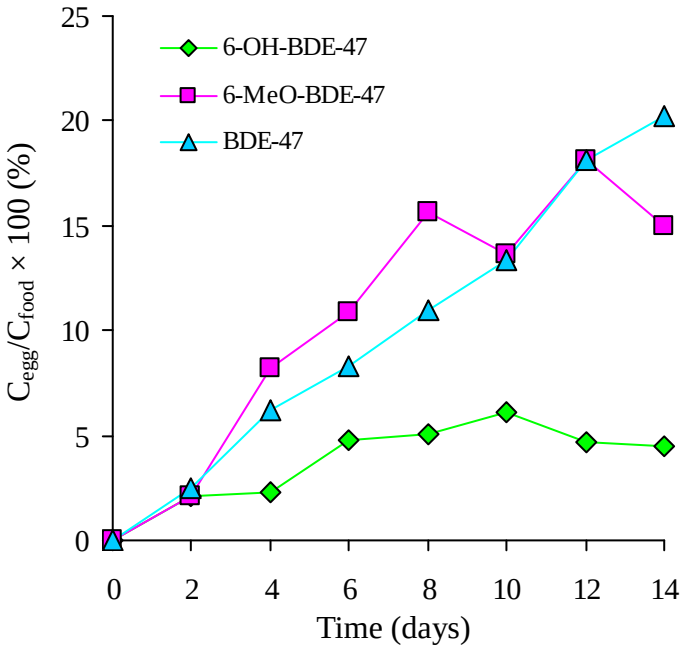

Figure 2.3 Accumulation of BDE-47, 6-OH-BDE-47, and 6-MeO-BDE-47 in eggs of female Japanese medaka during the course of a 14-day dietary exposure to feed spiked with either BDE-47, 6-MeO-BDE-47 or 6-OH-BDE-47. C_{egg} are the concentrations of target compounds in fish eggs, and C_{food} are the concentrations in dosing food. At each of the sampling time points all eggs from the individual experimental tanks were collected for analysis.

2.4 Discussion

2.4.1 Relationships among Parent Compounds and Their Transformation Products

Significant concentrations of 6-OH-BDE-47 were measured in medaka exposed to 6-MeO-BDE-47, but not BDE-47 (Figure 2.1a, Figure 2.2, and Table 2.3). Previous studies have indicated that an important prerequisite for exposure studies is ensuring standards and prepared exposure media are free of impurities that could lead to false results (Koistinen et al., 1996; Sanders et al., 2005). The analysis of purity suggested that 6-OH-BDE-47 was not a trace contaminant in any of the stock solutions or media employed in this study. Thus 6-OH-BDE-47 measured in fish and eggs from these treatment groups originated from

biotransformation of exposed parent compounds. This is consistent with results from our previous *in vitro* studies (Wan et al., 2009), further supporting the hypothesis that 6-MeO-BDE-47 is an significant contributor of 6-OH-BDE-47.

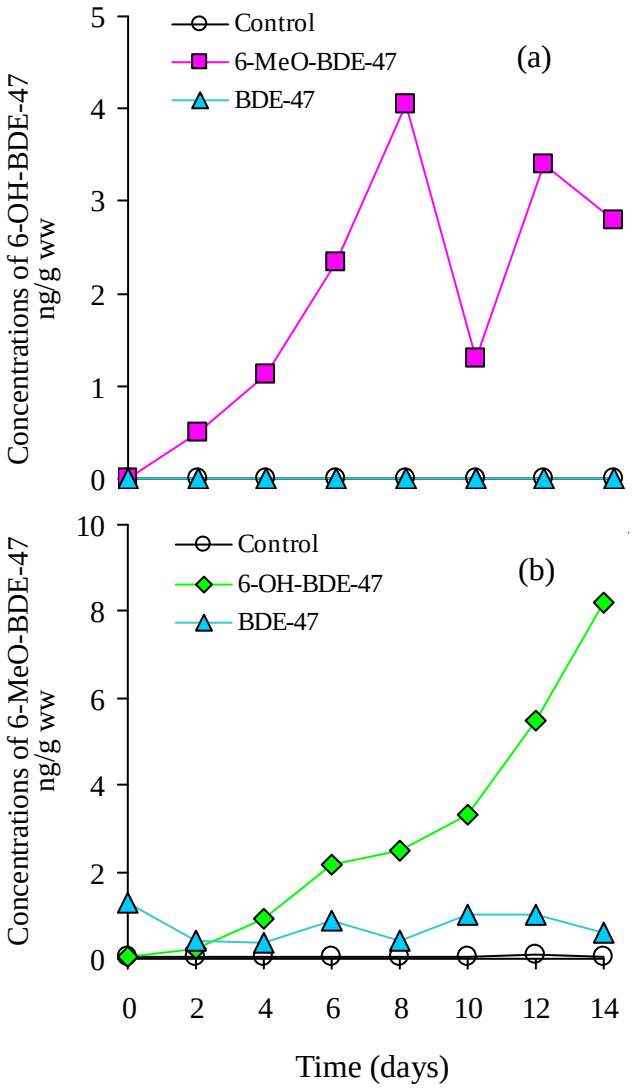

Figure 2.4 Accumulation trend of a) 6-OH-BDE-47 and b) 6-MeO-BDE-47 as metabolites in eggs of Japanese medaka during a 14-day dietary exposure to feed spiked with either solvent (control, panel a and b), BDE-47 (panel a and b), 6-MeO-BDE-47 (panel a) or 6-OH-BDE-47 (panel b). At each of the sampling time points all eggs from the individual experimental tanks were collected for analysis.

Table 2.3 Concentrations of target compounds after metabolism with medaka microsomes exposed to BDE-47, 6-MeO-BDE-47, and 6-OH-BDE-47 (ng/mL). The dosing concentrations for all chemicals were 2 μg/mL.

	Analyzed Chemicals		
Exposed Chemicals	BDE-47	6-MeO-BDE-47	6-OH-BDE-47
6-MeO-BDE-47	<1.6	710±72	62.8±9.9
6-OH-BDE-47	<1.6	<0.05	680±110
BDE-47	620±185	<0.05	<0.02

6-MeO-BDE-47 was also observed to be formed from 6-OH-BDE-47 in medaka. Detection of trace concentrations of 6-MeO-BDE-47 in all prepared food were not surprising (Table 2.1), since the ingredients of the commercial fish food include fish, plankton and shrimp, which could contain MeO-PBDEs of natural origin (Teuten et al., 2005). Assuming that the dosing level did not influence the food accumulation factors, the accumulation factors of fish exposed to parent 6-MeO-BDE-47 were used to calculate the concentrations of the impurity (6-MeO-BDE-47) accumulated in fish in other treatments. Concentrations of 6-MeO-BDE-47 in liver-free residual carcass were calculated to be 0.06±0.02 ng/g ww with dosing concentrations of 0.2 ng/g in food, which is similar to those of fish exposed to BDE-47 (0.1±0.03 ng/g ww) and in controls (0.06±0.02 ng/g ww). In addition, concentrations of 6-MeO-BDE-47 in medaka exposed to 6-OH-BDE-47 (liver-free residual carcass: 12.01±3.58 ng/g ww) were significantly greater than those in controls and medaka exposed to BDE-47 (p<0.01, Figure 2.1 b). A second feeding study was performed with 6-OH-BDE-47 in order to verify this observation. Consistent with the first study, significant concentrations of 6-MeO-BDE-47 compared to control group were observed. This result confirmed that 6-MeO-BDE-47 is formed as a biotransformation product of 6-OH-BDE-47. Previous studies have suggested that some MeO-PBDEs are formed via methylation of OH-PBDEs (Teuten et al., 2005; Haglund et al., 1997). The results presented here are the first experimental evidence of *in vivo* formation of MeO-PBDE from OH-PBDE. To further confirm this

observation, *in vitro* transformation of 6-OH-BDE-47 by microsomes isolated from female medaka was assessed (Table 2.3). Consistent with our previous *in vitro* studies (Wan et al., 2009), biotransformation of 6-OH-BDE-47 to 6-MeO-BDE-47 was not observed (Table 2.3). Taken together, 6-OH-BDE-47 can be transformed to 6-MeO-BDE-47 in Medaka, but the conversion does not occur in the hepatic microsomal fraction under the conditions of this experiment.

Comparable concentrations of BDE-47 were observed in female medaka exposed to 6-MeO-BDE-47 and 6-OH-BDE-47 (Figure 2.1c), which is likely due to BDE-47 impurities in the stock standard solutions. By using the calculation method above, concentrations of BDE-47 in liver-free residual carcass were calculated to be 7.0±1.9 ng/g ww and 2.7±1.0 ng/g ww with dosing concentrations of 28.3 and 15 ng/g ww in food, respectively. The calculated concentrations were comparable to those in fish exposed to 6-MeO-BDE-47 (liver-free residual carcass: 13.5±5.6 ng/g ww) and 6-OH-BDE-47 (9.4±10.0 ng/g ww). The fact that significant concentrations of neither 6-OH-BDE-47 nor 6-MeO-BDE-47 were detected in female medaka exposed to BDE-47 is consistent with some previous *in vitro* observations that some PBDE congeners are not biotransfromed into either OH-PBDEs or MeO-PBDEs in rainbow trout, Chinese sturgeon, salmon, chicken, beluga whale and rat microsomes (Zhang et al., 2010; Wan et al., 2009; Browne et al., 2009; McKinney et al., 2006a).

Significant differences in distributions of parent compounds and their biotransformation products were observed among tissues. Relatively large L/F ratios for 6-OH-BDE-47 are consistent with its preferential accumulation in liver compared to the other compounds studied. This result is also consistent with the tissue distribution of each target compound examined in Chinese green sturgeon (*Acipenser sinensis*), in which the greatest concentrations of 6-OH-BDE-47 were found in liver, while 6-MeO-BDE-47 and BDE-47

preferentially accumulated in adipose tissue (Zhang et al., 2010). The fact that the E/F ratio for 6-OH-BDE-47 was greater than the E/L ratio is also mainly due to the preferential accumulation of 6-OH-BDE-47 in liver. The greater maternal transfer ratios (E/L and E/F ratios) for 6-OH-BDE-47 as a parent compound than that of 6-OH-BDE-47 as a transformation product could be a result of the more than 10-fold lesser concentrations of 6-OH-BDE-47 formed as a transformation product. These results are consistent with reports that maternal transfer ratios of brominated flame retardants in zebrafish are dose-dependent, with greater ratios observed in individuals exposed to greater concentrations of the parent material (Nyholm et al., 2008).

2.4.2 Accumulation of Parent Compounds and Biotransformation Products in Eggs

Chemical-specific accumulation trends were observed for each parent compound over the course of the exposure period (Figure 2.3). In contrast to 6-OH-BDE-47, relatively great assimilation efficiencies were observed for 6-MeO-BDE-47 and BDE-47 as indicated by the steep slopes for accumulation during the initial 6 d of exposure. Accumulation of BDE-47 did not reach steady-state, but previous studies have reported that when zebrafish (*Danio rerio*) were exposed to BDE-47 eggs required 60 d to reach steady state (Nyholm et al., 2008). Based on the slow assimilation rate and large concentration ratios between fish and feed, it can be inferred that the depuration rate of BDE-47 is likely less than that of 6-MeO-BDE-47 (Tomy et al., 2004).

The observation that both 6-OH-BDE-47 and 6-MeO-BDE-47 occurred in eggs as biotransformation products of 6-MeO-BDE-47 and 6-OH-BDE-47, respectively, while neither transformation product was detected in eggs collected from medaka exposed to BDE-

47 is consistent with profiles of transformation products in liver and liver-free carcass (Figure 2.1). This observation is consistent with the hypothesis that OH-PBDEs are formed from MeO-PBDEs, not PBDEs. It is also consistent with OH-PBDEs being precursors of MeO-PBDEs *in vivo*. However, the time required to reach steady-state for 6-MeO-BDE-47 and 6-OH-BDE-47 as biotransformation products in eggs was different (Figure 2.4). The linear increase in concentration of 6-MeO-BDE-47 in eggs through biotransformation could be due to the great biotransformation ratios (M/P ratio) and/or maternal transfer of 6-MeO-BDE-47 as a transformation product (Table 2.4). Biotransformation products detected in eggs were likely generated in female medaka and subsequently transferred to the eggs. This conclusion is based on the fact that M/P ratios in medaka eggs were significantly less than those in liver and liver-free carcass. This result is consistent with the results of previous studies that have also demonstrated that the biotransformation capacity of fish eggs is less than later life stages (Nyholm et al., 2008; Petersen and Kristensen, 1998).

2.4.3 Naturally Occurring Concentrations and Relationships

Based on the results of the controlled laboratory study the relationship among PBDEs, OH-PBDEs, and MeO-PBDEs was further investigated by comparing concentrations in organisms collected worldwide (Table 2.4). When all the compounds were quantified, PBDEs, OH-PBDEs, and MeO-PBDEs were almost always co-detected in aquatic animals with BDE-47, 6-MeO-BDE-47, and 6-OH-BDE-47 as the predominant congeners. Concentrations of PBDEs have a wider range (0.2-2210 ng/g ww), and similar concentration variations were observed for MeO-PBDEs and OH-PBDEs. Concentrations of OH-PBDEs were generally greater than those of MeO-PBDEs in blood. This could be due to binding of OH-PBDEs to plasma transport proteins, including the thyroxine transport proteins (e.g.

transthyretin (TTR), thyroxine binding globulin (TBG)) (Malmberg et al., 2005; Meerts et al., 2000). The fact that both MeO-PBDEs and OH-PBDEs were detected with relatively great concentrations in liver suggested that liver will be the suitable tissue for investigations of the two groups of chemicals.

Relationships among PBDEs, OH-PBDEs and MeO-PBDEs in environmental samples are of great interest in context with the risk assessment of these compounds, especially the origin of the most toxic of these three classes of compounds, OH-PBDEs. Biotransformation of PBDEs to OH-PBDEs has been reported with quantified concentrations in previous *in vitro* (rat microsome and human-cell culture) (Hamers et al., 2008; Stapleton et al., 2009; Erratico et al., 2010) and *in vivo* (rat) (Malmberg et al., 2005) studies; however none of the studies have reported the purities of the standards used. In the *in vivo* exposure study, the concentration ratios of OH-PBDEs/PBDEs (M/P ratio) were very low (rat: 0.0002 and 0.004) (Malmberg et al., 2005) compared to those of current study (6-OH-BDE-47/6-MeO-BDE-47 ratio in liver of medaka: 0.021), and concentrations of detected OH-PBDEs remained constant even though concentrations of PBDEs decreased by more than ten times after five days of exposure (Malmberg et al., 2005). The possible contribution of impurities of MeO-PBDEs in commercial rat food should not be neglected, since 6-MeO-BDE-47 has been detected in the fish food in the current study. In the *in vitro* studies, the percentage of OH-PBDEs relative to PBDE dosing concentrations were <0.06%, 0.022-0.84% and 0.1-3% in BDE-99 exposed rat microsomes (Erratico et al., 2010), BDE-47 exposed rat microsomes (Hamers et al., 2008) and BDE-99 exposed human-cell culture (Stapleton et al., 2009), respectively. Relative greater percentages of 6-OH-BDE-47/6-MeO-BDE-47 were found in chicken (9%), rainbow trout (7%), rat (3%) and medaka (3%) liver microsome exposed to 6-MeO-BDE-47 (Wan et al., 2009) (Table 2.3). In addition, in our previous study (Wan et al., 2009), OH-PBDEs were not detected in the microsomes exposed to BDE-99, BDE-47 or

PBDE mixtures. In the current study no OH-BDEs were detected in Medaka exposed to BDE-47 *in vivo* or medaka microsomes exposed to BDE-47 *in vitro*. This is consistent with some previous in vitro observations that some PBDE congeners are not biotransfromed into OH-PBDEs in salmon, beluga whale and rat microsomes. Therefore, demethylation of MeO-PBDEs could be the primary source of ortho- substituted OH-PBDEs rather than hydration of PBDEs. A recent study with Chinese sturgeon also suggested that natural accumulation in the aquatic environment could be another important source of 6-OH-BDE-47 (Zhang et al., 2010). In addition, herein we report for the first time the *in vivo* biotransformation of 6-OH-BDE-47 to 6-MeO-BDE-47. The inter-conversion of 6-OH-BDE-47 and 6-MeO-BDE-47 in Japanese medaka was consistent with the co-detection of the two groups of chemicals in aquatic organisms.

Table 2.4 Reported concentrations of MeO-PBDEs and OH-PBDEs in blood and livers of various organisms worldwide. Concentrations are reported as ng/g.

Specie	Sample type	Location	References	Sex	MeO-PBDEs		OH-PBDEs		PBDEs	
					Mean	47(%)[a]	Mean	47(%)[b]	Mean	47(%)[c]
Glaucous gulls	Plasma	Norwegian Arctic	Verreault et al., 2005	M	0.95	5	0.43	33	20.2	44
				F	0.69	6	0.37	38	19.8	54
			Verreault et al., 2007a	M	1	-	0.44	-	21.3	-
				F	0.67	-	0.33	-	19.3	-
			Verreault et al., 2007b	-	2.78	-	3.54	-	51.5	-
Bald eaglet	Plasma	British Columbia, Canada	McKinney et al., 2006b	-	<0.01	-	0.31-0.92	40-100	1.78-8.49	43-53
Polar bear	Blood	East Greenland	Gebbink et al., 2008	-	0.16	70	2.9	<20	1.2	<40
Glaucous gulls	Liver	Norwegian Arctic	Verreault et al., 2007b	-	32.2	-	3.57	-	522	-
Albatross	Liver	Indian Ocean, South Atlantic Ocean, South Pacific Ocean	Wan et al., 2009	-	1.1	56	0.5	92	0.3	29
Polar bear	Liver	Arctic Ocean	Wan et al., 2009	-	0.02	9	0.01	63	0.7	83
Beluga whale	Liver	St. Lawrence River and Hudson Bay, Canada	Zhang et al., 2010	-	25	60-80	<0.5	>50	53	40
				-	43		<0.5		2210	
Tuna	Liver	North Pacific Ocean	Wan et al., 2009	-	0.5	69	0.03	84	0.2	38
Chinese sturgeon	Liver	Yangtze River	Zhang et al., 2010	F	0.03	74	0.2	83	22.7	57

a: average percentage of 6-MeO-BDE-47/ΣMeO-PBDEs; b: average percentage of 6-OH-BDE-47/ΣOH-PBDEs; c: average percentage of BDE-47/ΣPBDEs. Glaucous gulls (*larus hyperboreus*), polar bear (*Ursus maritimus*), bald eaglet (*Haliaeetus ieucocephalus*), beluga whale (*Delphinapterus leucas*), tuna (*Katsuwonus pelamis*), five albatross species (*Thalassarche chlororhynchos, Phoebetria palpebrata, T. chrysostoma, T. cauta, and T. melanophrys*), and Chinese sturgeon (*Acipenser sinensis*).

CHAPTER 3

MULTI-SPECIES COMPARISON OF THE MECHANISM OF BIOTRANSFORMATION OF MEO-BDES TO OH-BDES IN FISH

Abstract

Polybrominated diphenyl ethers (PBDEs) and their methoxylated- (MeO-) and hydroxylated- (OH-) analogs are ubiquitously distributed in the environment worldwide. In general, the OH-BDEs are more toxic than PBDEs and can be produced from the transformation of MeO-BDEs. The objectives of the current study were to 1) identify the enzyme(s) that catalyze the biotransformation of 6-MeO-BDE-47 to 6-OH-BDE-47 in livers of rainbow trout, and 2) compare transformation of 6-MeO-BDE-47 to 6-OH-BDE-47 among three species of fishes, namely rainbow trout, white sturgeon and goldfish. Cytochrome P450 1A (CYP1A) enzymes did not catalyze the transformation reaction. However, transformation was inhibited by the CYP inhibitors clotrimazole and 1-benzylimidazole but not gestodene. Therefore, the reaction is likely catalyzed by CYP2 enzymes. When transformation was compared among species, concentrations of 6-OH-BDE-47 were 3.4 and 9.1 times greater in microsomes from trout compared to goldfish and sturgeon, respectively. Concentrations of 6-OH-BDE-47 in microsomes from goldfish were 2.7 times greater than in sturgeon. The initial rate of transformation in microsomes from trout was 2.9 and 6.2 times greater than that of goldfish and sturgeon, respectively, while the initial rate in goldfish was 3.1 times greater than in sturgeon. It is hypothesized that differences in CYP mediated transformation of MeO-BDEs to OH-BDEs could influence concentrations of OH-BDEs in different species of fish.

Keywords: brominated, cytochrome P450, sturgeon, microsome, hydroxylation, inhibitor

3.1 Introduction

Polybrominated diphenyl ethers (PBDEs), used in a variety of consumer products, are ubiquitously distributed in the environment. In addition, structural analogs of PBDEs, such as the hydroxylated-BDEs (OH-BDEs) and the methoxylated-BDEs (MeO-BDEs) are also frequently measured in samples from the environment. Concentrations of OH-BDEs in organisms are sometimes greater than those of PBDEs (Unson et al., 1994; Malmvärn et al., 2005; Teuten et al., 2005; Covaci et al., 2007). For most toxicological endpoints the OH-BDEs are more potent than the corresponding PBDEs. This difference in toxicity is exemplified by differences in effects of OH-BDEs and PBDEs on the endocrine system. Several OH-BDEs, including 6-OH-BDE-47, have greater estrogenicity and androgenicity than PBDEs (Hu et al., 2011; Meerts et al., 2001; Hamers et al., 2008). OH-BDEs are structurally similar to thyroid hormones and have the ability to interact with thyroid hormone receptors (TR) (Kitamura et al., 2008; Kojima et al., 2009). OH-BDEs, but not PBDEs, significantly activate TRβ reporter gene expression in transactivation assays, and the most potent activator was 6-OH-BDE-47 (Li et al., 2010).

The OH-BDEs and MeO-BDEs have never been intentionally synthesized or used in manufacturing. Rather, those OH-BDEs and MeO-BDEs that have the -OH or -MeO group in the *ortho* position relative to the diphenyl ether bond, such as 6-OH-BDE-47 and 6-MeO-BDE-47, are produced naturally by marine organisms such as the marine sponge (*Dysidea herbacea*) or its associated filamentous cyanobacterium (*Oscillatoria spongeliae*), red alga (*Ceramium tenuicorne*), and green alga (*Cladophora fascicularis*) (Bowden et al., 2000; Fu et al.,1995; Handayani et al., 1997; Malmvärn et al., 2005, 2008). Although natural sources of the *para*- and *meta*- substituted OH-BDEs and MeO-BDEs have not been identified, potential for natural production cannot be excluded (Wiseman et al., 2011). In addition to being

natural products, it has been reported that OH-BDEs are formed by biotransformation of PBDEs (reviewed in Wiseman et al., 2011). For example, OH-BDEs were detected when human hepatocytes and microsomes isolated from livers of rats and beluga whales (*Delphinapterus leucas*) were exposed to PBDEs (Stapleton et al., 2009; Erratico et al., 2010; Hamers et al., 2008; McKinney et al., 2006a). However, transformation of PBDEs to OH-BDEs was not detected in a series of *in vivo* and *in vitro* studies (Wan et al., 2009; Wan et al., 2010; Stapleton et al., 2006; McKinney et al., 2006; Zhang et al., 2010). Recent studies have conclusively demonstrated that naturally occurring MeO-BDEs, and not synthetic PBDEs, are precursors of OH-BDEs (Wan et al., 2009; Wan et al., 2010; Wiseman et al., 2011). OH-BDEs were formed when microsomes isolated from livers of chickens (*Gallus gallus*), rainbow trout (*Oncorhynchus mykiss*) and rats (*Rattus norvegicus*) were exposed to MeO-BDEs (Wan et al., 2009). Accumulation of 6-OH-BDE-47 has also been reported in eggs and livers of Japanese medaka (*Oryzias latipes*) exposed to 6-MeO-BDE-47 through the diet (Wan et al., 2010).

Profiles of congeners of OH-BDEs and MeO-BDEs in wild fish vary among species (Valters et al. 2005; Covaci et al., 2008; Wang et al., 2011), and the accumulation patterns may be influenced by species-specific metabolism of MeO-BDEs, which has been observed for metabolism of PBDEs among different species (Roberts et al., 2011). Although *in vitro* and *in vivo* studies have demonstrated conversion of MeO-BDEs to OH-BDEs, the mechanism(s) of this transformation have not been elucidated. Therefore, the goal of the current study was to investigate the mechanisms of biotransformation of MeO-BDEs to OH-BDEs by use of livers from rainbow trout as a model system.

To this end the subcellular location of the biotransformation reaction, and the specific enzyme(s) involved in the transformation of MeO-BDEs to OH-BDEs were investigated. In

addition, differences in transformation of MeO-BDEs to OH-BDEs in different species of fish were investigated to determine whether differences in concentrations of OH-BDEs in different species could be due to differences in the transformation of MeO-BDEs to OH-BDEs.

3.2 Material and Methods

3.2.1 Chemicals

6-MeO-BDE-47 and 6-OH-BDE-47 were synthesized in the Department of Biology and Chemistry, City University of Hong Kong, Hong Kong, China. All compounds were determined to be >98% pure by high-resolution gas chromatograph interfaced to a high-resolution mass spectrometer (HRGC-HRMS). Dichloromethane (DCM), n-hexane, methyl tert-butyl ether (MTBE), acetone, acetonitrile (ACN) and methanol were pesticide residue grade and were obtained from OmniSolv (EM Science, Lawrence, KS, USA). Clotrimazole (CL), 1-benzylimidazole (BI), formic acid, hydrochloric acid (37%, A.C.S. reagent), 2-propanol, and silica gel (60-100 mesh size), and β-napthoflavone (βNF) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Gestodene (GE) was from Tecoland Corporation (Edison, NJ, USA). The anti-cytochrome P450 3A (CYP3A) antibody was a gift from Dr. Malin Celander (Department of Zoology, University of Gothenburg, Gothenburg, Sweden).

3.2.2 Test Organisms

Culturing of live fish was conducted in the Aquatic Toxicology Research Facility (ATRF) at the University of Saskatchewan's Toxicology Centre. This work was approved by

the University of Saskatchewan's Animal Research Ethics Board, and adhered to the Canadian Council on Animal Care guidelines for humane animal use. Sexually immature rainbow trout were from an in-house stock that was reared from eggs acquired from a commercial supplier (Troutlodge, Sumner, WA, USA). Sexually immature white sturgeon (*Acipenser transmontanus*) were from an in-house stock reared from eggs acquired from the Kootenay Trout Hatchery (Fort Steele, BC, Canada). Goldfish (*Carassius auratus*) were from a local pet supply store. Each species were maintained in separate 712 L tanks supplied with dechlorinated freshwater (municipal water). Rainbow trout and white sturgeon were maintained at 12 °C and goldfish were maintained at 20 °C, using a 12L:12D photoperiod. Rainbow trout were fed daily at approximately 2% of their body weight with commercial trout feed (Martin Classic Sinking Fish Feed, Martin Mills Inc., Elmira, ON, Canada). White sturgeon and goldfish were fed daily at approximately 2% of the bodyweight with frozen bloodworms (Hagen, Montreal, QC, Canada).

3.2.3 Subcellular Fractionation of Livers

Subcellular fractionation of livers was performed according to the microsome preparation methods of Kennedy and Jones (1994). All equipment were cleaned with nanopure water and rinsed with acetone and n-hexane prior to use. All procedures were performed on ice where possible and the equipment was chilled prior to use. Briefly, livers that were freshly excised were rinsed in ice-cold phosphate buffer (0.08 M sodium phosphate, 0.02 M potassium phosphate, pH 7.4), minced, and 100 to 200 mg of tissue was quantitatively transferred into a 2 ml microcentrifuge tube. Tissue was then homogenized with 10 strokes using a Fisher Scientific Powergen 125 (FTH-115) blade-type homogenizer, and the homogenate was centrifuged at 9,000 g in a SORVALL® Legend RT+ Centrifuge

(Thermo Fisher Scientific, Asheville, NC, USA) for 15 min at 4 °C. Next, a 100 µl aliquot of the supernatant from each sample, which represents the S9 fraction, was transferred to a 600 µl microcentrifuge tube and frozen at -80°C until needed. The remaining supernatant was transferred to ultracentrifuge tubes (SETON, Los Gatos, California, USA), and centrifuged at 100,000 g in a SORVALL® Ultraspeed Centrifuge (Thermo Fisher Scientific) for 60 min at 4 °C. The resulting supernatant (cytosol fraction) and pellet (microsomal fraction), which was re-suspended in 0.5 ml of ice-cold phosphate buffer, were stored at -80 °C. An aliquot of each preparation was used for determination of the concentration of protein using the BCA (bicinchoninic acid) method in 96 well plates according to the manufacturer's protocol (Sigma).

3.2.4 Localization of 6-OH-BDE-47 Formation

Transformation of 6-MeO-BDE-47 to 6-OH-BDE-47 was determined in S9, cytosol and microsome fractions. Reactions were performed in 250 µl of 0.05 M sodium phosphate buffer (pH 8.0) containing 10 mM DTT, and 0.5 mM NADPH, 10 µl of 6-MeO-BDE-47 (final concentration = 2 µg/ml), and an appropriate volume of the respective subcellular fraction to give a final concentration of 1.5 µg/µl protein were used in each reaction. The contents of the reaction tubes were allowed to warm to 25 °C after which reactions were initiated by addition of NADPH, a co-factor required by phase I transformation enzymes that are located in the S9 and microsomal fractions. Reactions were performed in the dark in an incubator at 25 °C for 4 h with constant rotation at 100 rpm. Incubations without proteins were used to investigate possible non-enzyme mediated chemical transformations. Once the reactions were completed the samples were stored frozen at -80 °C until extraction.

3.2.5 Co-factor Requirements for Formation of 6-OH-BDE-47

Microsomes isolated from livers of rainbow trout were used to determine if co-factors were required for biotransformation of 6-MeO-BDE-47 to 6-OH-BDE-47. Reactions were performed as described above without NADPH, DTT or without NADPH and DTT. Reactions containing both NADPH and DTT were performed as positive controls and incubations without proteins were performed as negative controls. Once the reactions were completed the samples were stored frozen at -80 ^{o}C until extraction.

3.2.6 Effect of AhR Activation on the Formation of 6-OH-BDE-47

To investigate whether phase I biotransformation enzymes induced by the activation of the aryl-hydrocarbon receptor (AhR) are involved in biotransformation of 6-MeO-BDE-47 to 6-OH-BDE-47, microsomes were isolated from livers of rainbow trout exposed to βNF (described in Doering et al., unpublished). Fish were injected intraperitoneally with either 50 mg/kg of βNF dissolved in corn oil or corn oil alone. Livers were collected three days after injection and were frozen in liquid nitrogen until use. Exposure to βNF activated AhR signaling, and EROD activity in livers from trout exposed to βNF was 510.6 compared to 13.9 pmol/min/mg protein in control trout (Doering et al., unpublished). Microsomes were isolated according the method described in section 3.2.3. Biotransformation reactions with 6-MeO-BDE-47 were performed as described in section 3.2.4.

3.2.7 Effects of Inhibitors of Cytochrome P450 on Formation of 6-OH-BDE-47

Specific and general inhibitors of cytochrome P450 (CYP) enzymes were used to determine the roles of specific families of CYP enzymes in transformation of 6-MeO-BDE-47 to 6-OH-BDE-47 (Table 3.1). Microsomes that were isolated from livers of rainbow trout were incubated with 2.5 µl of inhibitors dissolved in ethanol, or 5 µl of the anti-CYP 3A antibody. The final concentration of inhibitors was 0.1 mM. When microsomes were incubated with 2 inhibitors the final concentration of each inhibitor was 0.1 mM. Biotransformation reactions were performed as described in section 3.2.4 except inhibitors were added 30 min prior to the addition of the 6-MeO-BDE-47 and NADPH. After the reactions were completed samples were stored at -80 ^{o}C until analysis.

Table 3.1 Chemicals and antibodies, and the enzymes they target, to be used as part of the pharmacological approach to identifying the enzyme(s) responsible for the biotransformation of 6-MeO-BDE-47 to 6-OH-BDE-47.

Chemical/Antibody	Chemical/Antibody Description	Target CYP450 Enzyme
CYP 3A antibody	Anti-CYP 3A antibody	3A
Clotrimazole	Antifungal medication	1A, 2A, 2C,2E, 2K, 3A
1-Benzylimidazole	Inhibitor of thromboxane synthase	1A2, 2A, 2C, 2D, 2E, 2K, 3A
Gestodene	Progestogen hormonal contraceptive	3A

3.2.8 Species Comparison of the Formation of 6-OH-BDE-47

Microsomes were isolated form livers of rainbow trout, white sturgeon, and goldfish according to the protocol described above. Biotransformation reactions with 6-MeO-BDE-47 were performed as described above except that a NADPH regenerating system (BD

Biosciences, Mississauga, ON, Canada) was used to sustain concentrations of NADPH over the duration of the experiment. Microsomes were incubated with 6-MeO-BDE-47 for 0.5, 1, 2, 6 and 24 h. After reactions were completed samples were stored at -80 ^{o}C until use. To compare the initial rates of the transformation of 6-MeO-BDE-47 to 6-OH-BDE-47 the amount of product formed after 1 h, which corresponded to the linear portion of the time-course, was determined.

3.2.9 Determination of Concentrations of 6-OH-BDE-47

Quantification of 6-OH-BDE-47 was performed according to the methods of Wan et al. (2010) with minor modifications. Samples were spiked with 6'-OH-BDE-17 surrogates before extraction. A portion of 0.25 ml of 37% concentrated hydrochloric acid, 2 ml of nanopure water and 3 ml of 2-propanol were added, followed by addition of 3 ml n-hexane/MTBE (1:1 / v:v). This procedure was repeated 3 times to extract samples. The extracts were then washed 4 times with 2 ml of nanopure water to remove the residual acid and then the samples were concentrated and dried under a gentle stream of nitrogen gas. Next, 200 µl of aqueous sodium bicarbonate (100m mol/L, pH 10.5) and 200 µl of dansyl chloride (1 mg/ml in acetone) were mixed with the dried residues. The mixtures were incubated at 60 ^{o}C for 5 min and then vortex-mixed vigorously for 1 min. After cooling, 1 ml nanopure water and 3×3 ml of hexane were added to each sample. After vortex-mixing for 1 min, the upper layer of the mixtures was transferred onto silica gel columns, which were wet-packed with 4 g of silica gel (60-100 mesh size) and 4 g of sodium sulfate. A portion of 15 ml of hexane/DCM (1:1 / v:v), and 20 ml of DCM was used to elute the columns. The second fractions were rotary evaporated to near dryness at 35 ^{o}C and transferred to a tube by hexane/DCM (1:1 / v:v). Samples were then dried under nitrogen gas and reconstituted with

40 µl of ACN/water (6:4 / v:v) for liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis of 6-OH-BDE-47.

6-OH-BDE-47 was resolved using an XBridge C18 column (100 × 2.1 mm, 3.5 µm particle size) from Waters (Milford, MA, USA) and an Agilent 1200 series high performance liquid chromatography system (Santa Clara, CA, USA). The composition of the mobile phase (ACN / 0.1% formic acid in water) with a flow rate of 200 µl/min was maintained as 60:40 (v/v) from 0 to 1 min, which was increased to 95:5 / v:v from 1 to 15 min to generate a gradient elution. From 20 to 21 min, the ACN / 0.1% formic acid in water was then decreased to 60:40 / v:v and then equilibrated for 7 min. The 6-OH-BDE-47 was then detected by using an API 3000 triple-quadrupole tandem mass spectrometry (MS/MS) system (PE Sciex, Concord, ON, Canada), which was equipped with a turbo ion spray source operated in the positive multi-reaction monitoring (MRM) mode. 6-OH-BDE-47 was identified by comparison of the retention time and the mass/charge ratio with that of the authentic standard 6-OH-BDE-47. 6-OH-BDE-47 and internal standard 6'-OH-BDE-17 had mass/charge ratios of 735.9 and 658.1, respectively.

Concentrations of 6-OH-BDE-47 were quantified relative to authentic internal standard 6'-OH-BDE-17. Recovery of 6'-OH-BDE-17 was 88.9 ± 19.1% for all samples, and the concentrations of the analytes were recovery-corrected. All equipment was rinsed 3 times with acetone and hexane to prevent contamination during extraction, cleanup and instrumental analyses. For each batch of 12 samples, one laboratory blank sample was incorporated to monitor sample contamination. Since 6-OH-BDE-47 was not detected in all laboratory blanks, the method detection limits (MDL) were defined as the instrumental minimum detectable amounts. For those results that were less than the limit of detection, half of the MDL was assigned to avoid missing values in statistical analyses.

3.2.10 Statistical Analysis

All assays were performed in quadruplicate and results are reported as mean ± standard deviation (SD). Statistical analyses were performed using SPSS 17.0 (SPSS Inc., Chicago, IL, USA). Data were log-transformed where necessary to ensure homogeneity of variance. With the exception of the βNF exposure study, significant differences between or among experimental groups were evaluated by one-way analysis of variance (ANOVA). Significant differences between experimental groups in the βNF exposure study were evaluated by a two-sample t-test. Significant differences between experimental groups were assessed by the Tukey's post-hoc test. A p-value < 0.05 was considered significant.

3.3 Results and Discussion

3.3.1 Subcellular Localization of the Formation of 6-OH-BDE-47

The predominant pathway for generation of OH-BDEs is transformation of MeO-BDEs to OH-BDEs (Wan et al., 2009; Wan et al., 2010; Wiseman et al., 2011). However, the specific mechanism(s) of this biotransformation reaction were unknown to date. To determine the sub-cellular location of the transformation of 6-MeO-BDE-47 to 6-OH-BDE-47, reactions were performed with either S9, microsome or cytosolic fractions isolated from livers of rainbow trout (Figure 3.1). 6-OH-BDE-47 was not detected in the absence of any protein, which demonstrated that this reaction was catalyzed by an enzyme. 6-OH-BDE-47 was detected in the S9 and microsome fractions at mean concentrations of 22.9 and 178 ng/mg protein, respectively. The concentration of 6-OH-BDE-47 in the cytosolic fraction was less than the detection limit. Because the microsome and cytosolic fractions are components of the S9 fraction, the absence of 6-OH-BDE-47 in the cytosolic fraction

indicated that the specific enzyme(s) responsible for transformation of 6-MeO-BDE-47 to 6-OH-BDE-47 are localized to microsomes, and thus, are membrane-bound. Therefore, the statistically greater concentrations of 6-OH-BDE-47 formed in the microsome fraction were likely due to greater concentration of P450 enzymes that catalyze the transformation of 6-MeO-BDE-47 in this fraction compared to the S9 fraction.

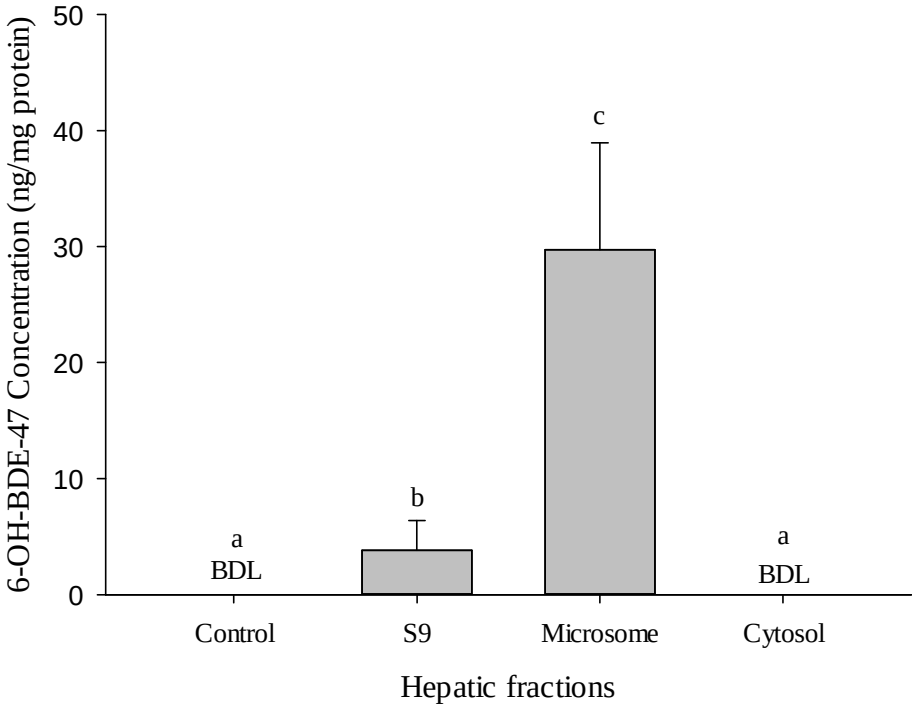

Figure 3.1 Concentrations of 6-OH-BDE-47 formed from the biotransformation of 6-MeO-BDE-47 in S9, microsome and cytosol fractions isolated form livers of rainbow trout. Bars represent mean ± SD of 4 independent replicates. Different letters above the bars represent statistically significant differences (one-way ANOVA with Tukey's post-hoc test; $p < 0.05$). (BDL= below detection limit).

3.3.2 Influence of Cofactors on Biotransformation of 6-MeO-BDE-47 to 6-OH-BDE-47

Microsomes isolated from liver cells contain phase I enzymes, such as cytochrome P450 enzymes (Rushmore and Kong, 2002). CYP450 enzymes require NADPH as a co-factor while cytosolic transformation enzymes, such as the deiodinases, require DTT as a

cofactor (Visser et al., 1982). To verify that phase I transformation enzymes catalyze transformation of 6-MeO-BDE-47 to 6-OH-BDE-47 reactions were performed with or without NADPH or DTT in the presence of microsomes isolated from livers of rainbow trout. Significantly greater concentrations of 6-OH-BDE-47 were detected in the presence of microsomes and incubated with NADPH and DTT, compared to reactions without either DTT or NADPH (Figure 3.2). However, concentrations of 6-OH-BDE-47 formed in the presence of microsomes incubated with only NADPH were significantly greater than in microsomes incubated in the presence of only DTT. 6-OH-BDE-47 was not detected in reactions where both NADPH and DTT (-NADPH / -DTT) were omitted from the reactions. Together, these results confirm that NADPH dependent phase I transformation enzymes catalyze transformation of 6-MeO-BDE-47 to 6-OH-BDE-47.

3.3.3 Effect of AhR Activation on Transformation of 6-MeO-BDE-47 to 6-OH-BDE-47

In rainbow trout, activation of the AhR signaling pathway by agonists such as βNF stimulates expression of multiple CYP1 enzymes, including greater activities of CYP1A1 and CYP1A2, but relatively lesser expression of CYP1B1, CYP1C1, CYP1C2, and CYP1C3 (Pesonen et al., 1987; Råbergh et al., 2000; Jönsson et al. 2010). To investigate possible involvement of AhR activation in transformation of MeO-BDEs to OH-BDEs, the transformation was quantified in microsomes isolated from livers of rainbow trout exposed to βNF. Exposure to βNF stimulated greater EROD (CYP1A1) and MROD (CYP1A2) activity in livers (Doering et al., unpublished). However, production of 6-OH-BDE-47 was not significantly greater in microsomes from livers of rainbow trout exposed to βNF than in microsomes from livers of control fish (Figure 3.3). These results suggest that P450 enzymes up-regulated by activation of AhR signaling, including the CYP 1A subfamily,

CYP1B1, CYP1C1, CYP1C2, and CYP1C3, do not catalyze transformation of 6-MeO-BDE-47 to 6-OH-BDE-47.

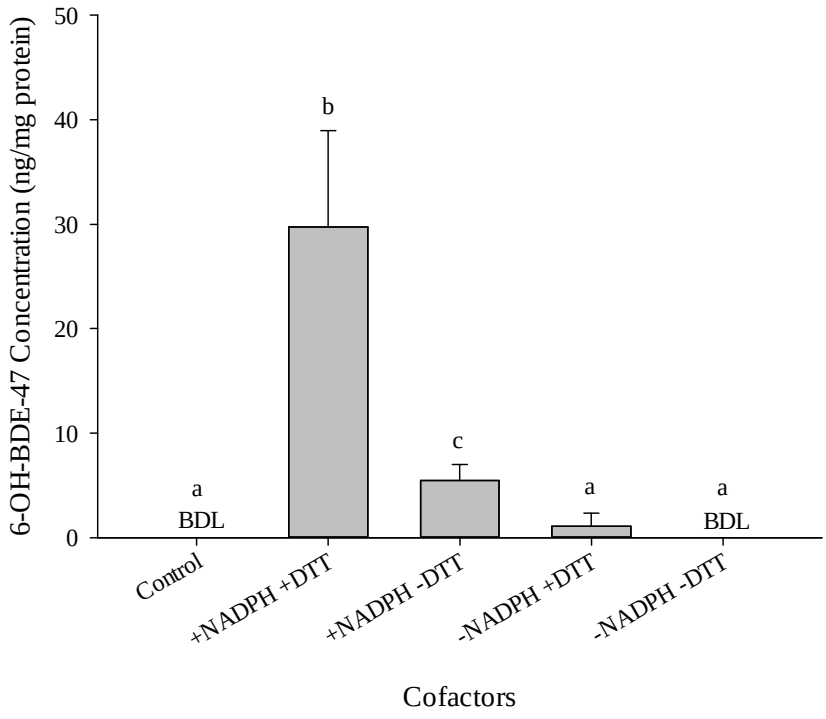

Figure 3.2 Effect of NADPH and DTT on the formation of 6-OH-BDE-47 from 6-MeO-BDE-47 in microsomes isolated form livers of rainbow trout. Bars represent mean ± SD of 4 independent replicates. Different letters above the bars represent statistically significant differences (one-way ANOVA with Tukey's post-hoc test; $p < 0.05$). (BDL= below detection limit).

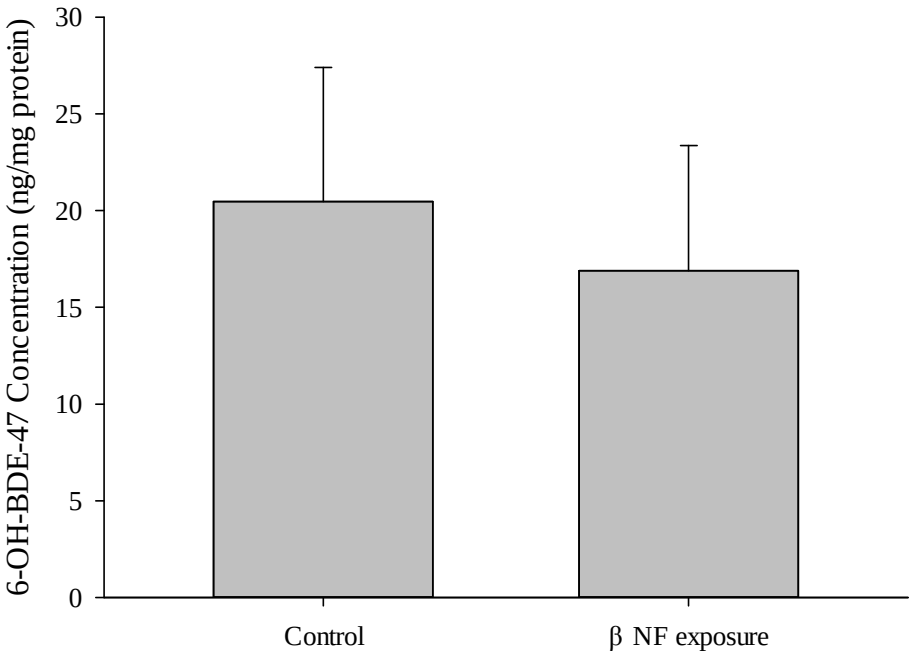

Figure 3.3 Concentrations of 6-OH-BDE-47 formed from the biotransformation of 6-MeO-BDE-47 in microsomes isolated from livers of rainbow trout IP injected with either corn oil (control) or corn oil containing 50 mg/kg bw of βNF. Bars represent mean ± SD of 4 independent replicates. Statistical difference was assessed by two sample t-test with $p < 0.05$

3.3.4 Effect of P450 Inhibitors on Transformation of 6-MeO-BDE-47 to 6-OH-BDE-47

Specific inhibitors of P450 enzymes were used to identify the enzyme(s) that catalyze transformation of 6-MeO-BDE-47 to 6-OH-BDE-47. The majority of xenobiotics are biotransformed by the P450 1, 2 and 3 families of enzymes (Siroka and Drastichova, 2004). Inhibitors of P450 enzymes have been characterized in mammalian systems and several of these inhibitors, including clotrimazole, 1-benzylimidazole and gestodene are effective as inhibitors of P450 enzymes from rainbow trout (Miranda et al., 1998). In this study, inhibition of transformation of 6-MeO-BDE-47 was specific to the type of inhibitor used (Figure 3.4). Exposure to clotrimazole, which is an inhibitor of CYP 1A, CYP2 (2A, 2C, 2E, 2K) and 3A enzymes (Ong et al., 2000; Rendic, 2002; Miranda et al., 1998), or 1-

benzylimidazole, an inhibitor of CYP1A2, CYP2 (2A6, 2C9, 2C19, 2D6, 2E1, 2K1) and CYP3 (3A4, 3A5, 3A27) enzymes (Grothusen et al., 1996; Miranda et al., 1998), significantly inhibited transformation of 6-MeO-BDE-47 to 6-OH-BDE-47 by 99 and 92%, respectively. When microsomes were co-exposed to clotrimazole and 1-benzylimidazole simultaneously, formation of 6-OH-BDE-47 was inhibited by 99.7%. In contrast, gestodene, a specific inhibitor of CYP3A enzymes but not CYP2K1 (Guengerich, 1990; Miranda et al., 1998), and the anti-CYP3A primary antibody decreased concentrations of inhibited the transformation of 6-MeO-BDE-47 to 6-OH-BDE-47 by 29% and 38%, respectively. These results, together with the results of βNF exposure study, suggest that transformation of 6-MeO-BDE-47 to 6-OH-BDE-47 is likely catalyzed by a CYP2 enzyme. However, biotransformation by CYP3A enzymes cannot be ruled out. Within the CYP2 superfamily, the CYP 2A, 2C, 2D and 2E enzymes have been identified in mammals to be inhibited by clotrimazole and 1-benzylimidazole. However, to date no comparable data is available for fish. The CYP2K subfamily is most abundant constitutively expressed, phase I enzymes in livers of rainbow trout (Williams and Buhler, 1984). CYP2K enzymes catalyse a series of hydroxylation reactions, including 2-hydroxylation of 17β-estradiol, 16α-hydroxylation of testosterone and progesterone, as well as ω-1 hydroxylation of lauric acid (Buhler and Wang-Buhler, 1998; Williams, 1984). This result suggests that CYP2K could catalyze transformation of 6-MeO-BDE-47 to 6-OH-BDE-47 in which a demethylation process is involved.

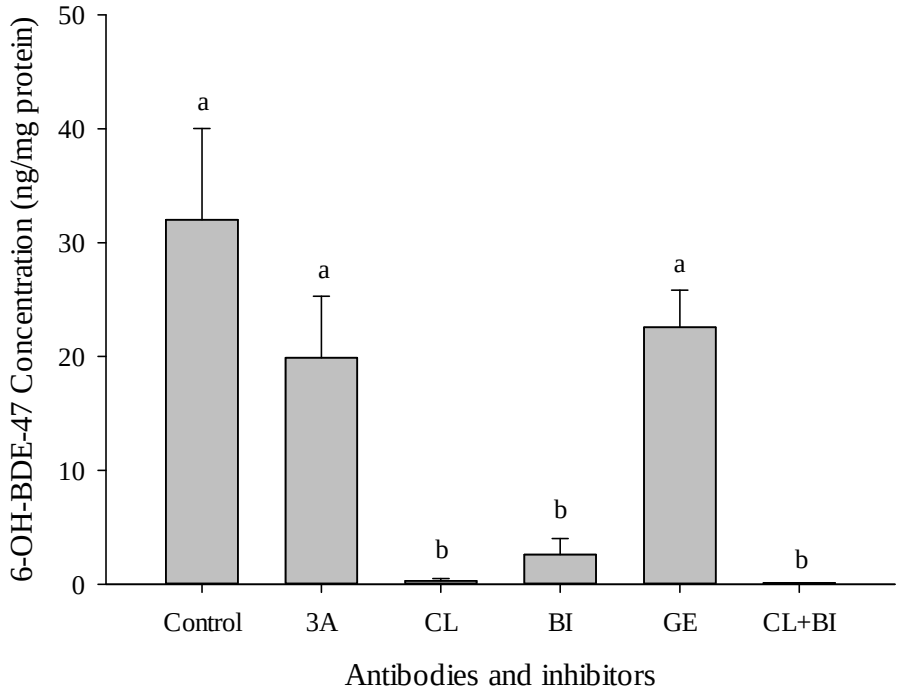

Figure 3.4 Effects of antibodies and inhibitors on the concentrations of 6-OH-BDE-47 formed from the biotransformation of 6-MeO-BDE-47 in microsomes isolated form livers of rainbow trout. A=CYP1A antibody; 3A=CYP3A antibody; CL = clotrimazole; BI = 1-benzylimidazole; GE = gestodene). Bars represent mean ± SD of 4 independent replicates. Different letters above the bars represent statistically significant differences (one-way ANOVA with Tukey's post-hoc test; $p < 0.05$).

3.3.5 Differences in Transformation of 6-MeO-BDE-47 to 6-OH-BDE-47 among Fishes

Debromination of PBDEs varies among species of fishes (Roberts et al., 2011). However, differences in the transformation of MeO-BDEs among species had never been investigated. Transformation of 6-MeO-BDE-47 to 6-OH-BDE-47 exhibited statistically significant differences among microsomes isolated from livers of rainbow trout, goldfish or white sturgeon (Figure 3.5). There was a time-dependent increase in the transformation of 6-MeO-BDE-47 to 6-OH-BDE-47 in all species but differences in the initial rate of the transformation were observed among the three species. The initial rate of transformation in

microsomes from livers of rainbow trout was significantly greater by 2.9- and 6.2-fold than the rate of transformation in microsomes from goldfish or white sturgeon, respectively. Similarly, the rate of transformation in microsomes from goldfish was significantly (3.1-times) greater than the rate of transformation in microsomes from white sturgeon. At the end of the 24h exposure the concentrations of 6-OH-BDE-47 were significantly different among microsomes isolated from livers of each species. Concentrations of 6-OH-BDE-47 in microsomes from livers of rainbow trout were significantly (3.4 times) greater than in microsomes from livers of goldfish and significantly 9.1 times greater than in microsomes from livers of white sturgeon. Concentrations of 6-OH-BDE-47 in microsomes from livers of goldfish were not significantly different from those in microsomes from livers from white sturgeon, but there was a trend towards greater (2.7-fold) concentrations in goldfish microsomes.

The specific activity or abundance of the P450 enzyme(s) that catalyze transformation of 6-MeO-BDE-47 to 6-OH-BDE-47 is likely greatest in microsomes isolated from livers of rainbow trout, followed by goldfish and white sturgeon. The results are consistent with previous studies where the transformation of 6-MeO-BDE-47 by microsomes from livers of Chinese sturgeon (*Acipenser sinensis*) liver (Zhang et al., 2010) was less than in microsomes from livers of rainbow trout (Wan et al., 2009). A number of studies have shown that P450 enzymes from different species of fish are neither expressed at the same levels nor have equal catalytic activities. For example, basal metabolism of 11 different substrates of CYP 1, 2 and 3 enzymes was greater in microsomes from livers from killifish (*Fundulus heteroclitus*) compared to rainbow trout (Smith and Wilson, 2010). Also, expression of CYP2K1 is different between gold-spotted trevally (*Carangoides fulvoguttatus*) and Stripey seaperch (*Lutjanus carponotatus*) inhabiting the Northwest Shelf of Australia (Zhu et al., 2008). Because the results of the current study suggested that a member of the CYP2 family, and

possibly CYP2K1, catalyzed transformation of 6-MeO-BDE-47 to 6-OH-BDE-47, it was hypothesized that the capacity for transformation of 6-MeO-BDE-47 to 6-OH-BDE-47 would be different among fishes.

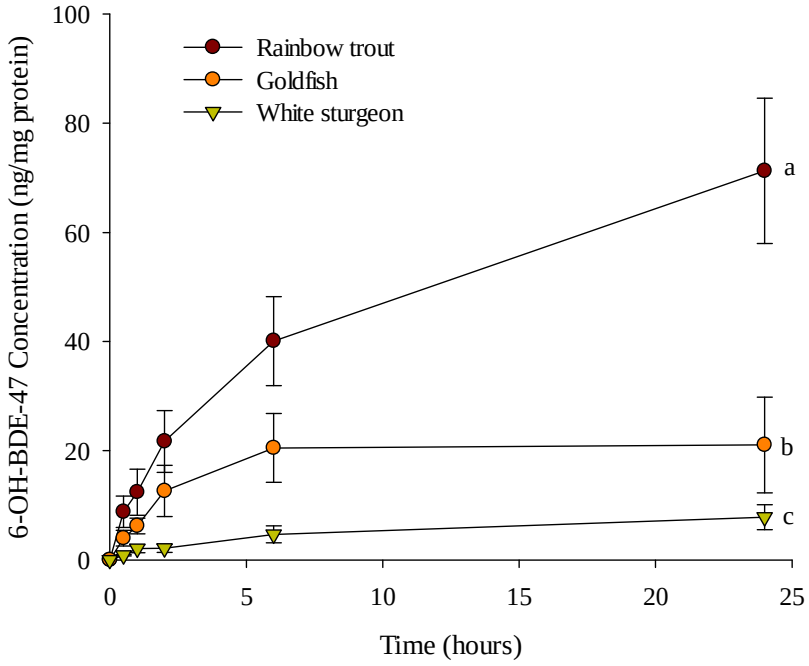

Figure 3.5 Time-course of the formation of 6-OH-BDE-47 from the biotransformation 6-MeO-BDE-47 in microsomes isolated from livers of rainbow trout, goldfish and white sturgeon. Bars represent mean ± SD of 4 independent replicates. Different letters represent statistically significant differences in the concentrations of 6-OH-BDE-47 at 24 h (one-way ANOVA with Tukey's post-hoc test; $p < 0.05$). Values for the amount of 6-OH-BDE-47 formed and the initial rates of the biotransformation reactions are given in Table 3.2.

Table 3.2 Initial rates of biotransformation and final concentrations of 6-OH-BDE-47 after a 24h exposure in microsomes isolated form livers of rainbow torut, gold fish and white sturgeon.

Species	**Initial Rate of Biotransformation (ng/mg protein/h ± SD)***	**Final Concentration 6-OH-BDE-47 (ng/mg protein ± SD)****
Rainbow trout	12.4 ± 4.2^{a}	$71.3 \pm 13.3^{*}$
Goldfish	6.2 ± 1.4^{b}	$21 \pm 8.8^{\S}$
White sturgeon	2.0 ± 0.7^{c}	$7.8 \pm 2.3^{\ddagger}$

Initial rates of biotransformation of 6-MeO-BDE-47 to 6-OH-BDE-47 were determined at 1h post initiation of the reaction.
* Values with different letters denote a significant difference in the rate of biotransformation (one-way ANOVA with Tukey's post-hoc test, $p<0.05$).
** Values with different symbols denote a significant difference in the final concentration of 6-OH-BDE-47 (one-way ANOVA with Tukey's post-hoc test, $p<0.05$).

Differences in the transformation of MeO-BDEs to OH-BDEs will affect concentrations of OH-BDEs in feral fishes. Concentrations of 10 OH-BDEs were different among 13 species of fish, including common carp (*Cyprinus carpio*), which along with the goldfish used in this study are members of the family Cyprinidae, and lake sturgeon (*Acipenser fulvescens*), collected from the Detroit River (Valters et al. 2005). The total concentrations of OH-BDEs and of 6-OH-BDE-47 were significantly greater in blood plasma from common carp compared to the lake sturgeon (Valters et al., 2005). Although MeO-BDEs were not detected in these fish samples, MeO-BDEs have been detected in sediment cores from two close by inland lakes (White Lake and Muskegon Lake) in the Great Lakes region of Michigan, USA (Bradley et al., 2011). Although differences in dietary intake levels of MeO-BDEs can be a factor of different concentrations of OH-BDEs in these species of fish, the results of the current study suggest that the greater concentrations of total OH-BDEs and 6-OH-BDE-47 in blood plasma from common carp compared to the lake sturgeon could be partly due to differences in the biotransformation of MeO-BDEs in these species. Further, the

failure to detect MeO-BDEs in these species may have been due, at least in some part, to their CYP2 mediated metabolism.

3.4 Conclusions

In conclusion, *in vitro* transformation of MeO-BDEs to OH-BDEs was confirmed for three fish species. The P450 enzyme-mediated transformation of 6-MeO-BDE-47 to 6-OH-BDE-47 appears to be mainly catalyzed by an enzyme of the CYP2 family. Furthermore, it was demonstrated that the degree of biotransformation of MeO-BDEs to OH-BDEs is dependent on species because of the different enzyme activities involved, which has implications for the risk assessment of these compounds. The results suggest that differences in concentrations of the more toxic OH-BDEs among wild fishes might be due to differences in the P450 catalyzed biotransformation of the naturally occurring MeO-BDEs. Species with greater biotransformaiton of MeO-BDEs to OH-BDEs could be exposed to greater concentration of OH-BDEs, which could lead to greater toxicity.

CHAPTER 4

GENERAL SUMMARY AND CONCLUSIONS

4.1 General Discussion

Polybrominated diphenyl ethers (PBDEs) and their hydroxylated (OH-), and methoxylated (MeO-) analogs are ubiquitous in the aquatic environment. The PBDEs are man-made chemicals used primarily as flame retardants. The OH-BDEs have never been intentionally produced, but it is important to understand the origin of OH-BDEs because, in general, they have greater toxicity than PBDEs (Meerts et al., 2001; Marsh et al., 1998). Although there was evidence that OH-BDEs are naturally occurring in the marine environment (Teuten et al., 2005), the results from several recent studies suggested that PBDEs might be precursors of OH-BDEs (McKinney et al., 2006a; Stapleton et al., 2009; Erratico et al., 2010; Kierkegaard et al., 2001). However, concentrations of OH-BDEs observed in studies of biotransformation of PBDEs were less than concentrations that would be expected to be formed based on concentrations of OH-BDEs in the environment. Also, it was found that trace impurities of OH-BDE in standard materials used in the studies of biotransformation could account for the observed OH-BDEs (Wan et al., 2009). Greater concentrations of OH-BDEs were quantified when microsomes from several species were incubated with MeO-BDEs *in vitro* (Wan et al., 2009). Those authors suggested that MeO-PBDEs are the primary precursors of the more toxicologically potent OH-BDEs (Wan et al., 2009).

To date, biotransformation of MeO-BDEs to OH-BDEs had not been investigated *in vivo*, and the mechanisms by which this biotransformation occurs had not been characterized.

To this end, a study of pathways of biotransformation among PBDEs, OH-BDEs and MeO-BDEs was conducted by use of Japanese medaka (*Oryzias latipes*). Furthermore, the enzymes involved in biotransformation of MeO-BDEs to OH-BDEs were investigated in subcellular fractions isolated form livers of rainbow trout (*Oncorhynchus mykiss*). Finally, comparisons of the capacity of different species of fish to transform MeO-BEs to OH-BDEs were determined. The results of these studies indicated that MeO-BDEs, but not PBDEs, are the precursor of OH-BDEs. Furthermore, the results of the study demonstrated that OH-BDEs are maternally transferred from adult, female medaka to their eggs. Biotransformation of MeO-BDEs to OH-BDEs appears to be catalyzed by a member (s) of the cytochrome P450 2 (CYP 2) family of enzymes. Differences in in transformation of MeO-BDEs to OH-BDEs among rainbow trout, goldfish (*Carassius auratus*) and white sturgeon (*Acipenser transmontanus*) were established.

4.2 Biotransformation Relationships between PBDEs, OH-BDEs and MeO-BDEs

Transformation relationships between BDE-47, 6-OH-BDE-47 and 6-MeO-BDE-47 were investigated in sexually mature, Japanese medaka. Fish were exposed through their diet to BDE-47, 6-OH-BDE-47 or 6-MeO-BDE-47 and concentrations of all compounds were quantified in livers and whole carcass (minus livers) of female fish as well as in eggs. Significant concentrations of 6-OH-BDE-47 were detected in medaka exposed to 6-MeO-BDE-47 via the diet. OH-PBDEs and MeO-PBDEs were not detected in fish exposed to the parent BDE-47. Similar results were demonstrated in eggs of medaka. In addition, as hypothesized, concentrations of BDE-47, 6-MeO-BDE-47 and 6-OH-BDE-47 in eggs increased during the exposure. Significant concentrations of 6-MeO-BDE-47 were also

detected in fish exposed to 6-OH-BDE-47 however, 6-MeO-BDE-47 was not detected when microsomes from livers of medaka were exposed to 6-OH-BDE-47.

It has been proposed that OH-BDEs in the environment result from products naturally produced by marine organisms and biotransformation from PBDEs by exposed organisms. However, the results presented in chapter 2 of this thesis, along with the results published by Wan et al. (2009), provided strong evidence that demethylation of naturally occurring MeO-BDEs is the main source of the more toxic OH-BDEs. As observed in the analysis of whole fish, reverse conversion from 6-OH-BDE-47 to 6-MeO-BDE-47 was demonstrated. This unusual observation could be a protective mechanism to reduce concentrations of the toxic OH-BDEs. As reviewed in chapter 1, it has been suggested that because of the binding affinity of OH-BDEs to the transport protein transthyretin (TTR), delivery of T4 to the developing fetus could be compromised, thereby leading to developmental effects. Using Japanese medaka, it was demonstrated that when fed MeO-BDEs in their diet, adult female medaka transfer OH-BDEs to eggs. Furthermore, because humans are exposed to naturally occurring MeO-BDEs via their diet, it is critical to determine whether maternal exposure to MeO-BDEs can cause accumulation of OH-BDEs in the fetus of humans and other organisms. Based on these results any risk assessment of OH-BDEs must include assessment of exposure to MeO-BDEs.

4.3 Mechanisms of Biotransformation of MeO-BDEs to OH-BDEs

Having conclusively demonstrated that OH-BDEs can result from biotransformation from MeO-BDEs, the goal of chapter 3 of this thesis was to characterize the mechanisms of biotransformation from 6-MeO-BDE-47 to 6-OH-BDE-47. Using subcellular fractions

isolated from livers of rainbow trout, significantly greater concentrations of 6-OH-BDE-47 were detected in microsomes compared to S9 fractions exposed to 6-MeO-BDE-47. This result suggests that biotransformation of 6-MeO-BDE-47 to 6-OH-BDE-47 is localized to microsomes. The involvement of NADPH as a co-factor further confirmed the involvement of phase I enzymes in this biotransformation. Non-significant transformation of 6-MeO-BDE-47 to 6-OH-BDE-47 was observed in microsomes isolated from livers of rainbow trout exposed to the aryl-hydrocarbon receptor (AhR) agonist βNF, which is an inducer of a suite of phase I biotransformation enzymes, including CYP1A1, CYP1A2, CYP1B1, CYP1C1, CYP1C2, and CYP1C3 (Pesonen et al., 1987; Råbergh et al., 2000; Jönsson et al., 2010), compared to unexposed trout indicated that neither of these enzymes catalyze this conversion. To further investigate which enzymes might be involved, the specific inhibitors of individual CYP enzymes, clotrimazole (CL), 1-benzylimidazole (BI) and gestodene (GE) and an anti-CYP 3A antibody were studied. Transformation of 6-MeO-BDE-47 to 6-OH-BDE-47 was significantly inhibited by the broad spectrum CYP450 inhibitors CL and BI. However, neither the CYP 3A inhibitor GE nor the anti-CYP 3A antibody altered the transformation rate significantly in microsomes exposed to 6-MeO-BDE-47. Based on all these results presented in chapter 3, transformation of 6-MeO-BDE-47 to 6-OH-BDE-47 is more likely to be catalyzed by a member of the CYP 2 subfamily.

Cytochrome P450 enzyme(s) catalyze the biotransformation of MeO-BDEs to OH-BDEs. The expression of many CYP450s is induced by exposure to natural and anthropogenic compounds. As a consequence, organisms that are exposed to inducers of CYP450s may have greater concentrations of OH-BDEs if they are exposed to MeO-BDEs via their diets. Because of the greater toxicity of OH-BDEs, this may have effects on organism health.

4.4 Species Comparison of Biotransformation from MeO-BDEs to OH-BDEs

Several studies have reported that P450 enzymes from different fishes are neither expressed at the same levels nor have equal catalytic activities (Smith and Wilson, 2010; Zhu et al., 2008). In addition, transformation of 6-OH-BDE-47 from 6-MeO-BDE-47 by microsomes isolated from livers of Chinese sturgeon (*Acipenser sinensis*) liver (Zhang et al., 2010) was significantly less than in microsomes from livers of rainbow trout (Wan et al., 2009). For the species comparison study the results of which are given in chapter 3, greater concentrations of 6-OH-BDE-47 were generated in microsomes isolated from livers of rainbow trout, followed by goldfish and white sturgeon. The initial rate of transformation for rainbow trout was significantly greater than that of goldfish and white sturgeon, while goldfish also had a significantly greater initial rate than did white sturgeon. The final metabolite concentration after 24 h exposure for rainbow trout was significantly greater than that of goldfish and white sturgeon. Similarly, the final concentration of 6-OH-BDE-47 for goldfish was also significant greater than white sturgeon.

Rainbow trout, goldfish and white sturgeon represent, respectively, a typical freshwater salmonid species, a temperate freshwater cyprinid, and an ancient bony fish species belonging to the Acipenseridae family. The different rates and capacity for biotransformation of 6-MeO-BDE-47 to 6-OH-BDE-47 might due to differences in abundances or specific activities of the enzyme responsible for this conversion. Different concentrations of OH-BDEs observed among fishes that have been reported previously (Valters et al., 2005; de la Torre et al., 2009; Van Bavel et al., 2001) could be due, in part, to differences in the biotransformation of MeO-BDEs in these fishes. Because the OH-BDEs have greater toxicity than PBDEs, different species exposed to the same concentration of MeO-BDEs may

be differentially impacted by this exposure due to differences in the capacity to biotransform MeO-BDEs to OH-BDEs.

4.5 Future Directions

The studies presented in this thesis have further expanded our knowledge of the origins of OH-BDEs in the environment. However, there are many still details of the relationships among PBDEs, MeO-BDEs and PH-BDEs that remain to be elucidated. For example, in chapter 2, a significant concentration of 6-MeO-BDE-47 was detected in Japanese medaka exposed *in vivo* to 6-OH-BDE-47, which was the first time that the transformation of OH-BDEs to MeO-BDEs has been reported. However, 6-MeO-BDE-47 was not detected when microsomes from livers of medaka were exposed to 6-OH-BDE-47. This suggests that tissue(s) other than liver, which is the primary tissue in the biotransformation of xenobiotic compounds, is responsible for this transformation reaction. Future studies should investigate this transformation reaction. CYP 2K1 expression in rainbow trout is sex-, organ- and age-related (Cok et al., 1998). For example, CYP 2K1 expressions in mature males were 2 and 25 times greater than that in liver and kidney of female fish respectively. Therefore, transformation of MeO-BDEs to OH-BDEs should be investigated in different tissues and in fish of different ages and different sexes capacities should be investigated. Furthermore, the basal enzyme activities of CYP 2K should be tested to determine if it is correlated with the biotransformation of MeO-BDEs to OH-BDEs in different species.

4.6 Conclusions

In conclusion, the studies described in this thesis demonstrated both biotransformation and maternal transfer from MeO-BDEs to OH-BDEs in Japanese medaka *in vivo*. Further, it was demonstrated, for the first time, that OH-BDEs can be transformed to MeO-BDEs *in vivo,* in non-hepatic tissue. The P450 enzyme-mediated transformation of 6-MeO-BDE-47 to 6-OH-BDE-47 appears to be mainly catalyzed by an enzyme of the CYP2 family. Significant differences in the *in vitro* transformation of MeO-BDEs to OH-BDEs was confirmed for three fish species, rainbow trout, goldfish and white sturgeon, which suggested that differences in concentrations of the more toxic OH-BDEs among wild fishes could, in part, be due to differences in a CYP 2 family enzyme catalyzed biotransformation of the naturally occurring MeO-BDEs.

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